UNIVERSITY OF ALBERTA

EFFECT OF UPSTREAM WATER TREATMENT PROCESSES ON AGGREGATION AND ULTRAVIOLET INACTIVATION OF *CRYPTOSPORIDIUM PARVUM*

by

FAISAL MAHMUD ©

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
in
ENVIRONMENTAL ENGINEERING

DEPARTMENT OF CIVIL AND ENVIRONMENTAL ENGINEERING

EDMONTON, ALBERTA

FALL 2006

Library and
Archives Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-22311-6
Our file Notre référence
ISBN: 978-0-494-22311-6

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

ABSTRACT

Upstream water treatment processes are likely to promote aggregation of *Cryptosporidium parvum* oocysts in filtered water. Aggregated oocysts might be shielded from UV irradiation and hence they may reduce the effectiveness of UV disinfection. In this study, untreated river water spiked with C. *parvum* oocysts was coagulated, flocculated, settled and then passed through a dual media filter column. Filtered water samples were exposed to UV irradiation using a collimated beam apparatus. A tissue homogenizer was used to disrupt the aggregates and disperse the oocysts before or after UV exposure. Oocyst inactivation was determined using a cell culture - foci detection method. The findings of the study suggested that under sub-optimal coagulation, a fraction of C. *parvum* oocysts that broke through the filter media were aggregated. This resulted in a significant reduction of UV inactivation of C. *parvum* oocysts in filtered water samples that were homogenized after UV exposure compared to the samples that were homogenized before UV exposure.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Stephen A. Craik and co-supervisor Dr. Miodrag Belosevic for their invaluable guidance and support

I would also like to acknowledge all the graduate students particularly Debbie Plouffe in Dr. Belosevic's lab who helped me tremendously with the parasitological work.

The technical assistance provided by Cezary Kucharski and Leah Gordon is greatly appreciated. A special thanks goes to fellow graduate students Dong Li and Rukevwe Dennis Uvbiama for their friendship, advice and technical support

The funding for this project has been provided by Alberta Ingenuity Centre for Water Research, the Natural Science and Research Council (NSERC) and the Canada Foundation for Innovation (CFI)

I am grateful to Margaret French and John Paran for their assistance in obtaining the raw water samples and the water quality data.

Finally, I would like to thank my wife, Tahmina and my family and friends for their encouragement, understanding and support.

DEDICATION

To the memory of my father, Akhlaqur Rahman for his
~~undying~~ love, spiritual guidance and moral support

TABLE OF CONTENTS

1.0 INTRODUCTION 1

1.1 BACKGROUND 1

1.2 PROBLEM STATEMENT 3

1.3 OBJECTIVES 3

1.4 RESEARCH APPROACH 4

1.5 ORGANIZATION OF THE THESIS 4

2.0 LITERATURE REVIEW 6

2.1 UV DISINFECTION OF DRINKING WATER 6

2.1.1 History 6

2.1.2 UV light 7

2.1.3 Mechanism of UV inactivation 8

2.1.4 UV light sources 9

2.1.5 UV dose Determination 11

2.1.6 Theory of Disinfection 14

2.1.7 Advantages and disadvantages of UV disinfection 14

2.2 BIOLOGY AND LIFE CYCLE OF *C. PARVUM* 15

2.2.1 Life cycle 16

2.3 UV INACTIVATION OF *C. PARVUM* 18

2.4 METHODS FOR DETECTING INFECTIVITY OF *C. PARVUM* 23

2.4.1 Cell Culture 24

2.4.2 Detection of Infectious *C. parvum* in cell culture 25

2.4.3 Comparison between Mouse infectivity and cell culture 27

2.4.4 Limitations of cell culture method 28

2.5 EFFECT OF PARTICLES ON UV DISINFECTION 29

2.5.1 Effect of upstream treatment processes on UV inactivation of *C. parvum* 31

2.6 NEED FOR THIS RESEARCH 34

3.0 EXPERIMENTAL MATERIALS AND METHODS 38
3.1 RAW WATER SAMPLE COLLECTION 38
3.2 WATER TREATMENT PROCESS SIMULATION 39
3.2.1 Coagulation, flocculation and settling 40
3.2.2 Filtration 42
3.3 UV EXPOSURE EXPERIMENTS 45
3.3.1 Collimated beam apparatus 45
3.3.2 Phosphate buffered water 46
3.3.3 Filtered water 47
3.3.4 Homogenization 48
3.3.5 Controls 49
3.4 *C. PARVUM* OOCYSTS PRODUCTION 49
3.5 IN VITRO CELL CULTURE 49
3.5.1 Cell growth and maintenance 50
3.5.2 Oocyst preparation 50
3.5.3 Cell infection 51
3.5.4 Foci Detection Method 52
3.6 INFECTIVITY ANALYSIS USING LOGISTIC DOSE-RESPONSE MODEL 54
3.7 IMPACT OF HOMOGENIZATION ON THE INFECTIVITY OF *C. PARVUM* OOCYST 55
3.8 DIRECT MEASUREMENT OF OOCYST AGGREGATION 55
3.9 MICROSCOPIC EXAMINATION OF OOCYST AGGREGATION 56
3.9.1 Scanning Electron microscopy (SEM) 56
3.9.2 Confocal microscopy 57
4.0 ANALYSIS OF THE RESULTS 58
4.1 LOGISTIC DOSE RESPONSE MODEL 58
4.2 IMPACT OF HOMOGENIZATION ON THE INFECTIVITY OF *C. PARVUM* OOCYST 60
4.3 WATER TREATMENT PROCESS SIMULATION EXPERIMENTS 61
4.4 UV EXPOSURE EXPERIMENTS 64
4.4.1 Phosphate buffered water 65

4.4.2 Filtered water........67
4.4.3 Controls........72
4.5 DIRECT MEASUREMENT OF OOCYTS AGGREGATION........73
4.6 MICROSCOPIC EXAMINATION OF OOCYSTS AGGREGATION........76
4.6.1 Scanning Electron Microscopy (SEM)........76
4.6.2 Confocal Microscopy........78

5.0 DISCUSSION........83

5.1 WATER TREATMENT PROECESS SIMULATION........83
5.2 HOMOGENIZATION........84
5.3 INFECTIVITY ASSAY........84
5.4 UV EXPOSURE EXPERIMENTS........86
5.5 SERIAL FILTRATION AND MICROSCOPY........88

6.0 CONCLUSIONS AND RECOMMENDATIONS........90

6.1 CONCLUSIONS........90
6.2 RECOMMENDATIONS........91

APPENDIX A INFORMATION FROM LOGISTIC DOSE RESPONSE EXPERIMENTS OF *CRYPTOSPORIDIUM PARVUM* OOCYSTS .. 102

APPENDIX B INFORMATION FROM THE FILTRATION RUNS........105

APPENDIX C INFORMATION FROM THE UV EXPOSURE EXPERIMENTS 116

APPENDIX D STATISTICAL ANALYSIS........120

APPENDIX E DATA FROM CUT-OFF FILTRATION EXPERIMENTS AND IMAGES FROM SCANNING ELECTRON AND CONFOCAL MICROSCOPY........126

LIST OF TABLES

Table 2-1 Summary of experimental conditions adapted in different studies involved in developing UV dose response relationship for C. *parvum*. ... 21

Table 3-1 Water quality characteristics of the raw water samples collected in July, 2005 and November, 2005. ... 38

Table 3-2 Alum and polymer dose used for coagulation in different filtration runs42

Table 3-3 Physical properties of the filter media ... 44

Table 3-4 Typical flow rates at different points of the filtration run ... 44

Table 3-5 Experimental conditions of different filtration runs ... 44

Table 4-1 Logistic dose response models of C. *parvum* oocysts in cell culture ... 60

Table 4-2 Effect of homogenization on the infectivity of C. *parvum* oocysts ... 61

Table 4-3 Turbidity, pH and temperature of water measured at different point during filtration run #4 ... 62

Table 4-4 Results from UV exposure trials for oocysts suspended in phosphate buffered water ... 66

Table 4-5 Results from UV exposure trials for oocysts collected in filtered water from filtration run no. 3 ... 68

Table 4-6 Results from UV exposure trials for oocysts collected in filtered water from filtration run no. 4. ... 69

Table 4-7 Results from UV exposure trials for oocysts collected in filtered water from filtration run no. 5 ... 71

Table 4-8 Results from control trials carried out along with the UV exposure trials ... 73

Table A-1 Oocysts dose-response data for oocysts batch no. 1 ... 103

Table A-2 Oocysts dose-response data for oocysts batch no. 2 ... 104

Table B-1 Turbidity, pH and temperature of water measured at different point during filtration run #1....107

Table B-2 Turbidity, pH and temperature of water measured at different point during filtration run #2....108

Table B-3 Turbidity, pH and temperature of water measured at different point during filtration run #3....110

Table B-4 Turbidity, pH and temperature of water measured at different point during filtration run #4....111

Table B-5 Turbidity, pH and temperature of water measured at different point during filtration run #5....112

Table B-6 Turbidity, pH and temperature of water measured at different point during filtration run #6 (November '05 sample)....114

Table B-7 Turbidity, pH and temperature of water measured at different point during filtration run #6 (July '05 sample)....114

Table C-1 Information from UV exposure trials for oocysts suspended in phosphate buffered water. 117

Table C-2 Conditions of the UV exposure trials for oocysts collected in filtered water from filtration run no. 3. 118

Table C-3 Conditions of the UV exposure trials for oocysts collected in filtered water from filtration run no. 4. 118

Table C-4 Information from UV exposure trials for oocysts collected in filtered water from filtration run no. 5. 119

Table D-1 Summary output of ANOVA for filtered water samples exposed at 5 mJ/cm^2....121

Table D-2 Summary output of ANOVA for filtered water sample exposed at 40 mJ/cm^2....124

Table E -1 Data from cut-off filtration experiment........127

Table E-2 Oocysts recovery from each sample........128

LIST OF FIGURES

Figure 2.1 The electromagnetic spectrum of UV light 7

Figure 2.2 Approximate absorption spectrum of DNA 8

Figure 2.3 UV emission of spectra of (a) LP and (b) MP Lamps 10

Figure 2.4 Schematic diagram of a collimated beam apparatus 11

Figure 2.5 Divergence factor 13

Figure 2.6 C. *parvum* life cycle in host cells 16

Figure 2.7 UV dose response curves of C. *parvum* obtained from published peer reviewed studies 22

Figure 3.1 Jar testing apparatus used for simulation of coagulation, flocculation and settling 41

Figure 3.2 Schematic diagram of the simulated filtration process 43

Figure 3.3 Collimated beam apparatus used for UV exposure experiments 45

Figure 3.4 Tissue homogenizer 48

Figure 3.5 Confocal microscopic images of infected and non-infected cell monolayers... 53

Figure 4.1 Cell culture logistic dose-response model for oocysts from batch #1 59

Figure 4.2 Cell culture logistic dose-response model for oocysts from batch #2 59

Figure 4.3 Turbidity of the filtered water as function of time for filtration run no.4. 63

Figure 4.4 Particle concentration of the filtered water as function of time for filtration run no. 4 63

Figure 4.5 UV inactivation of C. *parvum* oocysts observed in different studies using low pressure lamps.. 67

Figure 4.6 Percent of total recovered oocyts retained on PCTE filters with different pore size 74

Figure 4.7 SEM micrograph of a large floc particle surrounded by C. *parvum* oocyst-like particles which might have been detached from the surface of the floc particle. The pore size of the PCTE filter was 5 μm. 77

Figure 4.8 SEM micrograph showing large floc particle (approximately 30 μm) captured in a 5 μm pore size PCTE filter 77

Figure 4.9 SEM micrograph showing oocyst-like particles attached to other particles captured on a the surface of a 10 μm pore size PCTE filter 78

Figure 4.10 DIC-IFA image of aggregated C. *parvum* oocysts captured from filtered water. Oocysts appear to be associated with large floc particles 79

Figure 4.11 DIC-IFA image of a floc particle captured from filtered water. C. *parvum* oocysts appear to be embedded on the floc particle surface. 80

Figure 4.12 DIC-IFA image of a floc particle captured from filtered water. C. *parvum* oocysts appear to be embedded within the floc particle 80

Figure 4.13 DIC-IFA image of a C. *parvum* oocyst clump captured from filtered water. 81

Figure 4.14 DIC-IFA image of a C. *parvum* oocyst clump captured from filtered water. 81

Figure 4.15 DIC-IFA image of C. *parvum* oocysts that were captured from a homogenized filtered water sample. 82

Figure B-1 Tracer test on filter column using methylene blue 106

Figure B.2 Turbidity of the filtered water as function of time for filtration run #1 107

Figure B.3 Particle concentration of the filtered water as function of time for filtration run #1 108

Figure B.4 Turbidity of the filtered water as function of time for filtration run #2 109

Figure B.5 Particle concentration of the filtered water as function of time for filtration run #2 109

Figure B.6 Turbidity of the filtered water as function of time for filtration run #3 110

Figure B.7 Turbidity of the filtered water as function of time for filtration run #4..111

Figure B.8 Particle concentration of the filtered water as function of time for filtration run #4....................112

Figure B.9 Turbidity of the filtered water as function of time for filtration run #5..113

Figure B.10 Particle concentration of the filtered water as function of time for filtration run #5....................113

Figure B.11 Turbidity of the filtered water as function of time for filtration run #6..114

Figure B.12 Particle concentration of the filtered water as function of time for filtration run #6....................115

Figure D.1 Normal probability plot of residuals for ANOVA at 5 mJ/cm^2............122

Figure D.2 Plot of residuals vs estimates for ANOVA at 5 mJ/cm^2......................122

Figure D.3 Plot of residuals vs filtration run for ANOVA at 5 mJ/cm^2.................123

Figure D.4 Plot of residuals vs sample type for ANOVA at 5 mJ/cm^2..................123

Figure D.5 Normal probability plot of residuals for ANOVA at 40 mJ/cm^2...........124

Figure D.6 Plot of residuals vs estimates for ANOVA at 40 mJ/cm^2.....................125

Figure D.7 Plot of residuals vs sample type for ANOVA at 40 mJ/cm^2................125

Figure E.1 SEM micrograph showing a large particle captured on a 5 µm pore size PCTE filter from backwash water....................128

Figure E.2 SEM micrograph showing particles captured on a 10 µm pore size PCTE filter from backwash water....................129

Figure E.3 SEM micrograph of isolated *C. parvum* oocyst captured on a 0.8 µm pore size PCTE filter....................129

Figure E.4 SEM micrograph of a floc particle with *C. parvum* oocysts-like particle attached to the surface of the floc particle. 130

Figure E.5 DIC-IFA image of aggregated *C. parvum* oocysts captured from filtered water....................130

Figure E.6 DIC-IFA image of a floc particle captured form filtered water.............131

Figure E.7 DIC-IFA image of a particle captured form filtered water...................131

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
CO_2	Carbon Dioxide
DNA	Deoxyribonucleic Acid
DI	Deionized water
ELISA	Enzyme-Linked Immunosorbent Assay
FDM	Foci Detection Method
FITC	Fluorescein Isothiocyanate
ID_{50}	Parasite dose required for 50% rate of infection
LT2SWTR	Long term 2 Enhanced Surface Water Treatment Rule
mRNA	Messenger RNA
N/A	Not Applicable
NOM	Natural Organic Matter
NTU	Nephelometric Turbidity Units
PCR	Polymerase Chain Reaction
PBS	Phosphate buffer saline
PVC	Polyvinyl Chloride
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RNA	Ribonucleic Acid
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
UVDGM	Ultraviolet Disinfection Guidance Manual

LIST OF SYMBOLS

a	Absorption coefficient
d_0	Total number of oocysts in inoculum to each well
d	Estimated number of infectious oocysts in each inoculum
D	UV dose
DF	Divergence factor
E_0	Incident irradiance
E_g	Gemicidal irradiance
L_d	Distance from UV lamp to the liquid surface
L	Value of likelihood function
N_0	Total number of life oocysts before UV exposure
N	Total number of live oocysts after UV exposure
k	First order rate constant
L	Sample depth
$N_i(\lambda)$	Relative photon flow in a given wavelength band
P	Proportions of wells infected
p	Probability of type I error
PF	Petri factor
n	Number of samples
RF	Reflection factor
t	Exposure time
WF	Water factor
X_i	Log (base 10) of the inoculum size for each well in dose response
Y_i	binary score for each well (0 or 1) in dose response

Greek Symbols

β_0, β_1	Parameters of the logistic dose-response model
λ	Wavelength

1.0 INTRODUCTION

1.1 BACKGROUND

Cryptosporidium parvum is an obligate, intracellular protozoan parasite that infests the gastrointestinal tract of humans and animals causing severe diarrhea illness. *C. parvum* is ubiquitous in surface water sources around the world and has been reported to cause many waterborne outbreaks (Mackenzie et al., 1994; Craun et al., 1998; Fayer et al., 2000; Schuster et al., 2005) . In 1993, *C. parvum* caused a massive waterborne outbreak in Milwaukee, Wisconsin affecting over 400,000 people (Mackenzie et al., 1994). An estimated 5,800 to 7,100 people experienced gastrointestinal cryptosporidiosis because of a *C. parvum* outbreak in North Battleford, Saskatchewan (PHAC, 2001). Traditional disinfectants, i.e. chlorine has been proved to be ineffective against these parasites at the concentration and contact times practical for water treatment plants (Korich et al., 1990). Furthermore, chlorination leads to the formation trihalomethans (THMs) and haloacetic acids (HAAs) as disinfection by products (DBPs) which are known to have deleterious health effects. Ozone is effective against *C. parvum*, but it also has the potential to form carcinogenic DBPs, bromate in the presences of bromine and organic DBP precursors in raw water. Ultraviolet technology has been recently proven to be very effective against these parasites (Bukhari et al., 1999; Clancy et al., 2000; Craik et al., 2001; Mofidi et al., 2001; Shin et al., 2001). Significant log inactivation of *C. parvum* was achieved even at UV doses (Clancy et al., 2000) ranging from 3-9 mJ/cm^2. UV light penetrates the oocysts wall and damages the DNA, preventing them from replicating further. Therefore, in order to achieve proper inactivation, the suspension of oocysts being irradiated has to be clean and the UV light reaching the microorganisms must be uninterrupted. If the oocysts being irradiated are attached to particles or other oocysts, they might be shielded from UV light and the overall log inactivation will potentially be overestimated.

In a conventional water treatment plant, particles in the raw water are coagulated, flocculated and settled prior to filtration. Coagulation and flocculation helps to increase the tendency of small particles in aqueous suspension to attach to one another and create large flocs. These pre-treatment processes promote aggregation of particulate matter and microorganisms so that they can be readily removed by granular media filtration. The performance of these processes plays an important role in removing *C. parvum* oocysts in filtered water. Many waterborne outbreaks have been related to sub-optimal operation of the coagulation/flocculation/settling/filtration processes (Mackenzie et al., 1994; Craun et al., 1998; PHAC, 2001). In the Milwaukee outbreak, difficulties with the flocculation procedure led to production of higher turbidity water and may have allowed *Cryptosporidium* oocysts to break through the filtration barrier (Mackenzie et al., 1994). Similarly, in North Battleford, Saskatchewan, the sub-optimal efficiency of the solid contact unit upstream of the filtration could potentially have allowed oocysts to pass into the finished drinking water. Some of the oocysts that break through the filters during periods of sub-optimal filter operation or upset conditions are likely to be aggregated with other oocysts or particulate matter. These oocysts can potentially escape UV disinfection even at high applied dose. Turbidity and particle counts are often used as indicators of oocysts removal in filtered water. However, several studies have demonstrated that turbidity and particle counts are indicative of overall treatment performance but not of oocysts removal (Lechevallier and Norton, 1992; Nieminski and Ongerth, 1995; Huck et al., 2002). Huck et al. (2002) observed substantial deterioration of *C. parvum* oocyst removal even at turbidity levels <0.3 NTU when the coagulation was not optimized. Turbidity is a measure of light scattering of the suspended particles present in water and hence it does not provide any direct information regarding the particle association or aggregation of the microorganisms.

1.2 PROBLEM STATEMENT

The USEPA's Long Term II Enhanced Surface Water Treatment Rule (LT2ESWTR) recognizes ultraviolet (UV) disinfection as an acceptable technology for primary disinfection of C. *parvum* (USEPA, 2006). According to the LT2ESWTR, an applied UV dose of 12 mJ/cm^2 is required for 3 log inactivation of C. *parvum* oocysts in filtered water. This criterion is based on the outcomes of several studies in which C. *parvum* oocysts were spiked into relatively clean water and then exposed to UV irradiation. The effect of aggregation of the oocysts on UV inactivation was not considered in those studies. Aggregated oocysts might be shielded from UV light and this phenomenon might reduce the overall UV inactivation achieved. Several studies have evaluated the effect of particle association of microorganisms on UV disinfection in filtered drinking water (Christensen and Linden, 2003; Batch et al., 2004; Passantino et al., 2004; Amoah et al., 2005). In most of the studies, C. *parvum* oocysts were spiked into filtered or artificial water with elevated turbidity levels and their log inactivation following UV exposure was determined. Spiking studies have several drawbacks. Firstly, the oocysts are unlikely to associate with other particles or oocysts without an external influence i.e. coagulation/flocculation, secondly the nature and the state of particle association of the oocysts in the spiked water might be different from those which break through the treatment system during periods of sub-optimal operation. Therefore, there is need to investigate the degree and nature of aggregation of C. *parvum* oocysts that passed through the conventional water treatment processes under sub optimal operation and subsequently to determine the effect on UV inactivation.

1.3 OBJECTIVES

The principal objectives of the study are:

- To determine the extent of aggregation of C. *parvum* oocysts that break through a simulated water treatment processes during periods of suboptimal coagulation,
- To determine the UV inactivation of aggregated C. *parvum* oocysts and compare with the UV inactivation of free, monodispersed oocysts

1.4 RESEARCH APPROACH

A lab scale experimental study composed of following steps was used to achieve the above mentioned objectives.

Water treatment processes simulation - Simulate the conventional water treatment process at bench-scale using a jar testing apparatus and a dual sand media filter column for coagulation/flocculation/settling and filtration respectively.

UV exposure – Expose the filtered water to low pressure UV irradiation using a collimated beam apparatus. Use a tissue homogenizer to break up the oocyst – particle and oocysts – oocysts aggregation either before or after the UV exposure.

Infectivity analysis – Determine the loss in infectivity of C. *parvum* oocysts following UV exposure using a cell culture based infectivity assay.

Direct measurement of particle association – Pass the filtered water through a series of membrane filters with decreasing pore size and enumerate the number of oocysts retained on each filter.

Microscopic observation of particle association – Use scanning electron microscopy and confocal micorscopy to examine the aggregated oocysts captured from filtered water.

1.5 ORGANIZATION OF THE THESIS

This thesis is organized into five chapters. Chapter 1 provides general background information and states the objectives of the thesis. Chapter 2 discusses the pertinent literature on this topic. The methods adopted and the materials used to accomplish the research objectives are presented in Chapter 3. Results obtained from the study and the analysis of results is provided in Chapter 4. Chapter 5 contains a summary discussion about the results how they relate to the objectives and previous work and finally, chapter 6 includes the conclusions drawn from the research and some recommendations for future work. All the raw data generated during the course of the study and are provided in the Appendix.

2.0 LITERATURE REVIEW

This chapter reviews relevant literature on ultraviolet (UV) disinfection of drinking water, biology and life cycle of C. *parvum*, UV inactivation of C. *parvum*, methods available for analyzing infectivity of C. *parvum* and the effect of particle association on the UV inactivation of C. *parvum*. At the end of this chapter, the need for this research is discussed.

2.1 UV DISINFECTION OF DRINKING WATER

2.1.1 History

The disinfection capability of UV light was first reported by Downes and Blunt (1887), when they observed the effect of sunlight radiant energy on bacteria. However, the UV part of the sunlight that reaches the earth's surface is merely confined to wavelengths higher than 290 nm. The first large scale application of UV technology in drinking water was at Marseilles, France in 1910 (USEPA, 2003). In the United States, the first full-scale application of UV light started in 1916 at Henderson, Kentucky (Masschelein, 2002). Several other treatment plants in Europe and North America were also reported to use UV disinfection at that time. However, all these applications were abandoned in the late 1930s. The reasons were unknown but presumably costs, maintenance of the equipment and aging of the lamps were determinants. Disinfection with chlorine was preferred because of easy operation and lower cost at that time(USEPA, 2003). Up to 1980, the information on the use of UV in the United States was anecdotal. The EPA Surface Water Treatment Rule (SWTR) of 1989 did not indicate UV as the best available technology for inactivation of *Giardia lamblia* (USEPA, 1989). The proposed Groundwater Disinfection Rule (GWDR) (USEPA, 2000) however, included UV as a possible technology. Since 1990, joint research efforts have been made by American Water Works Association (AWWA) and the AWWA Research Foundation (AWWARF). In 1998, it was demonstrated that UV could be appropriate for inactivation of C. *parvum* oocysts (Clancy et al., 1998). This was supported by other researchers who also established the UV sensitivity of C. *parvum* (Bukhari et al., 1999; Clancy et al., 2000; Craik et al., 2001; Mofidi et al., 2001; Shin et al., 2001). These findings together with increasing public concern about the disinfection by product (DBP) formed by chlorination encouraged USEPA to include UV disinfection in the proposed Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) (USEPA, 2006) for additional treatment of C. *parvum*.

2.1.2 UV light

UV light is the region of the electromagnetic spectrum that lies between x-rays and visible light (Figure 2.1). The spectrum can be divided into four ranges: vacuum UV (100 to 200 nm), UV-C (200 to 280 nm), UV-B (280 to 315 nm), and UV-A (315 to 400 nm) (Meulemans 1986). The UV-A range causes tanning of the skin while the UV-B range causes the skin to burn and is known to eventually induce skin cancer. The UV-C range is so-called "germicidal range" since it is absorbed by proteins, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), and can lead to cell mutations and/or cell death. It is therefore, effective in inactivating pathogens. The vacuum UV range is so powerful that it is absorbed by almost all substances including water and air. Typically, the practical germicidal wavelength for UV light ranges between 200 and 300 nm, i.e. essentially UV-C and UV-B (Bolton, 2001).

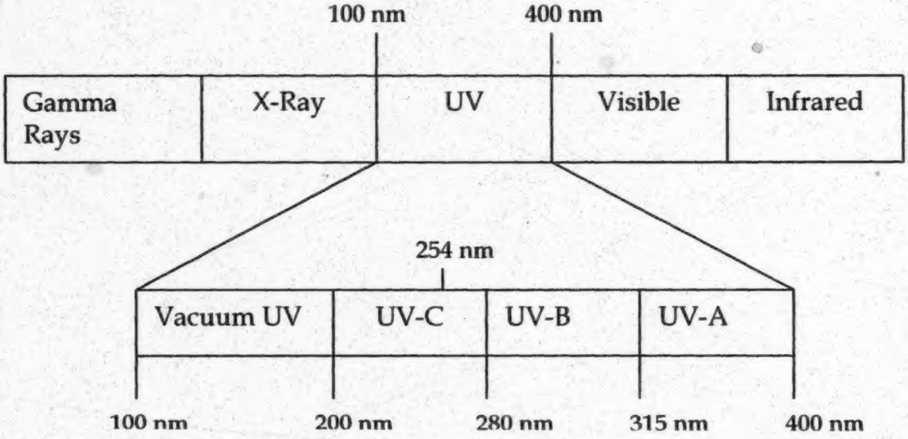


Figure 2.1 **The electromagnetic spectrum of UV light (adapted from USEPA, 2003)**

2.1.3 Mechanism of UV inactivation

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are the building blocks of most living cells. DNA and RNA are comprised of combinations of four bases. In DNA, bases are purines (adenine and guanine) and pyrimidines (thymine and cytosine). In RNA, the purines are the same as DNA but in case of pyrimidines, thymine is replaced by uracil. In a double stranded nucleic acid, bases on one strand compliments those on the other strand. Adenine pairs with thymine in DNA (or uracil in RNA) while guanine pairs with cytosine. Hydrogen bonds form between each pair (USEPA, 2003). These bases in DNA and RNA absorb UV light with a range of 200 to 300 nm (Harm, 1980). The UV adsorption of DNA is a combination of absorption of theses bases and has a peak at about 260 nm (figure 2.3).

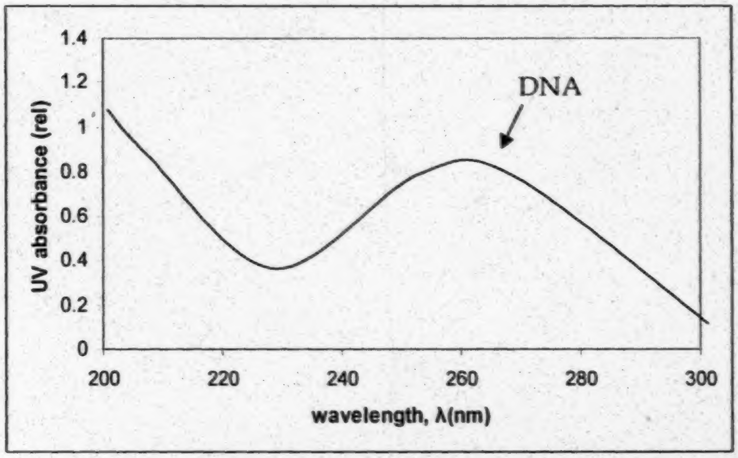


Figure 2.2 Approximate absorption spectrum of DNA (adapted from Bolton, 2001)

UV disinfection is a physical process, which inactivates waterborne microorganism via photochemical changes to their nucleic acids preventing their replication. Direct damage to the nucleic acid is the primary mechanism of UV light inactivating microorganisms. When UV energy is absorbed by the genetic material of microorganisms, thymine bases of the double stranded DNA undergo a unique photochemical reaction. If two thymine bases are located adjacent to each other, absorption of UV light by one of the thymines leads to formation of a chemical bond between the two thymine, eventually forming a thymine dimer (Bolton, 2001). This results in damaging the structure of DNA and if enough thymine dimers are formed, the DNA cannot replicate in cell miotosis. In case of microorganisms which contain only RNA (i.e. virus), the dimerization reaction takes place between two uracil bases. Microorganisms with DNA rich in the thymine tend to be more sensitive to UV disinfection (USEPA, 2003). Although microorganisms with damaged DNA are still viable, they cannot replicate and thereby cannot cause infection to the host. Variation in DNA/RNA structure can cause the microorganisms to absorb UV light differently which may lead to variation in inactivation rate. Among the pathogens of interest in drinking water, viruses are most resistant to UV disinfection followed by spore-forming bacteria, vegetative bacteria , *Cryptosporidium* oocysts and *Giardia* cysts.

2.1.4 UV light sources

Activation (or ionization) of mercury atoms by electrons (i.e., electrical discharges) is the most popular technology for generating UV light for drinking water disinfection at present. Activation-ionization by collision with electrons and return to a lower energy state is the principle of production of light in the mercury discharge lamps. The reasons for the preference for mercury are that it is the most volatile metal element for which activation in the gas phase can be obtained at temperatures compatible with the structures of the lamps. Moreover, it requires the least amount of energy to produce UV light (Masschelein, 2002). The most common lamps that are used in drinking water disinfection are low pressure (LP), low pressure high output (LPHO) and medium pressure (MP) mercury arc lamps.

2.1.4.1 Low pressure (LP) and low pressure high output (LPHO) lamps

Low-pressure (LP) mercury lamps used for the generation of UV light are normally operated at a nominal total gas pressure in the range of 102 to 103 Pa (0.01 to 0.001 mbar) and at a moderate temperature (40 °C). LP lamps emit monochromatic light. The principal emission of LP lamps is at 253.7 nm which is close to the maximum wavelength at which DNA and RNA absorb UV light. The output of the LP lamps is around 40 W for a 122 cm lamp and they have a lifetime of 8000-10000 hours (Bolton, 2001). Placement of a small amount of another metal (e.g., indium or gallium) that forms an amalgam on the inner surface of a LP UV lamp allows the lamp to operate at a much higher power. Thus, the output of a LPHO UV lamp is 2 - 3 times higher per unit length than that of LP UV lamps (Bolton, 2001). Similar to the LP lamps, LPHO lamps have a monochromatic emission at 253.7 nm and a lifetime >8000 hours. The use of these lamps is growing in popularity because these lamps share most of the advantages of LP lamps but with a higher output.

2.1.4.2 Medium pressure lamps

The medium pressure mercury lamp operates at a total gas pressure range of 10 to 30 MPa (1 to 3 bar). Medium pressure lamps emit polychromatic UV light with wavelength ranging from 200 to 400 nm (Figure 2.4). Although, MP lamps have lower efficiency (10 to 15%) compared to LP and LPHO lamps (35 to 45%), they have a high output (150 times higher than LP and 30 times higher than LPHO). This means proportionally fewer lamps will be required to disinfect a given water stream with MP lamps when compared to LP or LPHO lamps. However, the power requirement of MP lamps is very high (1 to 25 KW per lamp) and this may result in increasing the overall operating cost of the UV disinfection system significantly.

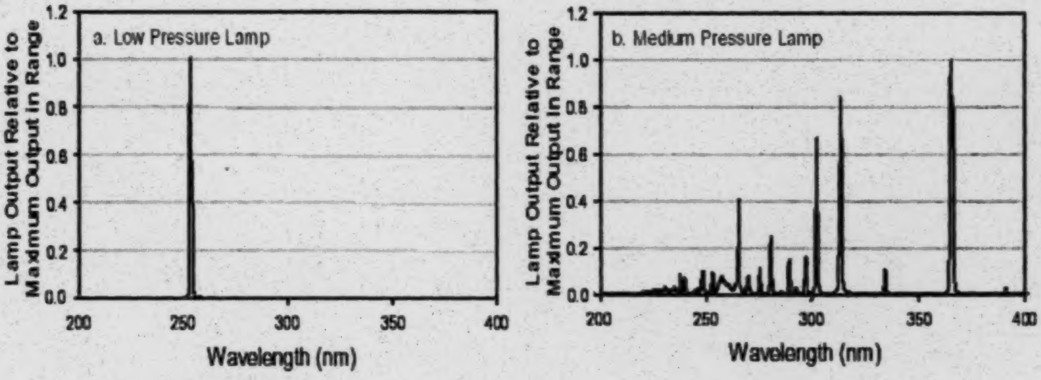


Figure 2.3 UV emission of spectra of (a) LP and (b) MP Lamps (USEPA, 2003)

2.1.5 UV dose Determination

2.1.5.1 Collimated beam apparatus

Bench scale UV experiments are typically conducted using a collimated beam apparatus. A schematic diagram of the collimated beam apparatus is provided in Figure 2.4. A UV lamp (LP or MP) is mounted in the upper cabinet and is separated from the collimator tube by a pneumatically driven shutter. The UV collimator is a long tube, which makes sure that the UV rays reaching the suspension of microorganism in the Petri dish are reasonably parallel and uniform. The inside walls of the UV collimator are painted black, so that any UV light that hits the walls is not reflected back to the tube. The distance from the lamp to the surface of water in the Petri dish is an important for better collimation of the UV beam. Generally, the greater the distance, the better the collimation will be.

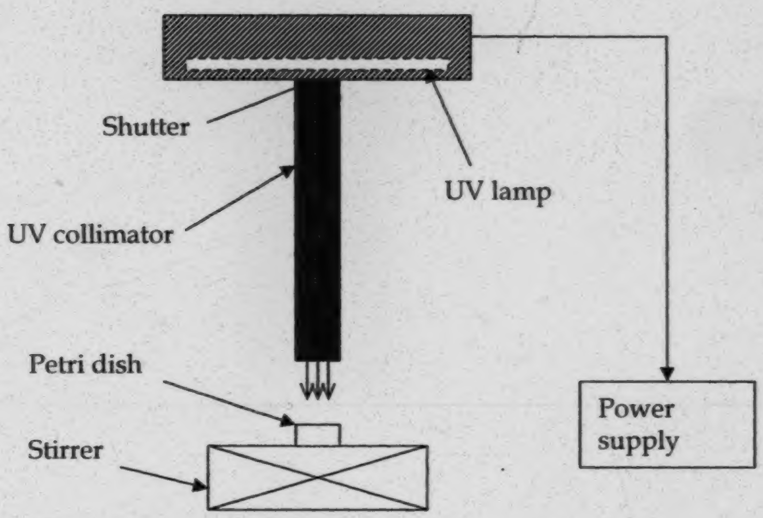


Figure 2.4 Schematic diagram of a collimated beam apparatus

2.1.5.2 Dose measurement

Irradiance is the term used for describing intensity of uni-directional UV light on a flat surface. *Irradiance* is defined as the total radiant power incident on an infinitesimal element of surface area dA containing the point under consideration divided by dA (Bolton, 2001). The incident *irradiance*, E_0 (mW/cm2) of the collimated beam is measured at the liquid surface in the centre point of the Petri dish. The radiometer sensor reading which directly gives the *irradiance* is averaged over the depth of the water in Petri dish, *l*. This depth averaged *irradiance* is multiplied by the following factors to determine the true irradiation across the Petri dish.

Reflection factor

When the incident beam hits the water surface in the Petri dish, a part of it is reflected back from the interface between air an water. According to the Fresnel's law, 2.5% of the incident beam is reflected (R) and the *reflection factor* (*RF* = 1-R) is calculated as 0.975.

Sensor factor

The UV detector in the radiometer has a sensitivity that varies with wavelength. *Sensor factor* (*SF*) is the sensitivity at 254 nm divided by the weighted average sensitivity of the detector over the range of wavelength emitted from the lamp (Equation 2.1).

$$SF = \frac{S_{254}}{\bar{s}}; \; \bar{s} = \frac{\sum_i N_i(\lambda) s_i(\lambda)}{\sum_i N_i(\lambda)} \tag{2-1}$$

Where, $N_i(\lambda)$ = relative photon flow in a given wavelength band. *Sensor factor* is only important for polychromatic UV lamps (i.e. MP) which have a broadband emission. For LP lamps, sensor factor is considered to be 1.0 (Bolton and Linden, 2003).

Petri factor

The *Petri factor* (*PF*) accounts for the non uniformity of UV irradiance across the Petri dish. Petri factor is the irradiance averaged over the cross section of the Petri dish divided by the centre irradiance. The Petri factor can be determined by methodically scanning the radiometer detector over the area of the Petri dish, dividing the irradiance at each point by the centre irradiance and taking the average of these ratios (Bolton and Linden, 2003).

Water Factor

Water Factor (*WF*) corrects for the attenuation of the incident irradiance due to the absorption of UV light in the irradiated water column. In a completely mixed sample, WF is determined from an integrated form of the Beer–Lambert law,

$$WF = \frac{1-10^{-al}}{al\ln(10)} \tag{2-2}$$

Where, a is the absorption coefficient of the solution (cm^{-1}) at the wavelength of irradiation and l is the vertical path length (cm) of the water in the Petri dish (Bolton and Stefan, 2002).

Divergence factor

The UV beam incident on the water surface of the Petri dish is not perfectly collimated and diverges significantly if the distance between the lamp and the water surface is small (Bolton and Linden, 2003). The irradiance at L_d+x (Figure 2.6) relative to that at the distance L is,

$$\frac{L_d^{\ 2}}{(L_d+x)^2} \tag{2-3}$$

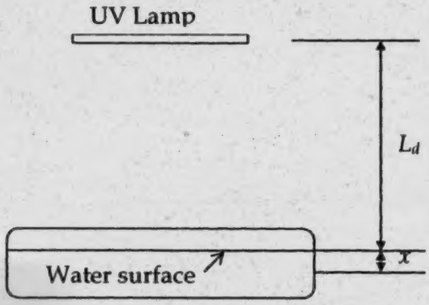


Figure 2.5 Divergence factor

The *divergence factor* (*DF*) is the average of this function over the path length, l of the suspension in the Petri dish.

$$DF = \frac{L_d}{(L_d+l)} \tag{2-4}$$

For a low pressure UV lamp, the depth averaged germicidal irradiance, E_g (mW/cm^2) can be calculated as,

$$E_g = E_0 \times PF \times RF \times WF \times DF \tag{2-5}$$

For a suspension of microorganism that has been irradiated for t sec, the UV dose or fluence, D (mJ/cm^2) can be estimated as,

$$D = E_g \times t \tag{2-6}$$

2.1.6 Theory of Disinfection

The simplest inactivation kinetic model for UV irradiation is a mixed first order expression similar to the Chick-Watson law often used to describe the kinetics of microbial inactivation by chemical disinfectants (Gyürék et al., 1997):

$$\frac{dN}{dt} = -kE_g N \tag{2-7}$$

Where, k is the first order rate constant, N is the number of survivors, E_{avg} is the average germicidal irradiance and t is the time.

The integrated from of equation (7) is,

$$\frac{N}{N_0} = e^{-kE_g t} \tag{2-8}$$

Where, N_0 and N are the number of viable microorganisms in the suspension before and after UV exposure.

2.1.7 Advantages and disadvantages of UV disinfection

The following are the advantages and disadvantages of UV disinfection:

Advantages

- UV disinfection has been proven to be very effective against protozoan and some viruses which are not readily inactivated by chlorine at a reasonable concentration and contact time.
- No carcinogenic by products have been detected at the dose typically applied in drinking water.
- No toxic residues are created in treated water.
- Small footprint and very little contact time required.
- No hazard from handling, transporting or storage of chemicals such as chlorine.

Disadvantages

- High UV doses are required to inactivate some viruses.
- Interferences by particles present in water and wastewater which can shield the microorganism from UV inactivation.
- Uncertainties in measuring UV doses.
- Biofilm formation or chemical scaling on lamp surfaces.

- Sometimes the DNA or RNA damaged by UV irradiation is repaired by the microorganism with or without the presence of light.
- No residual for long term disinfection in the distribution system.

2.2 BIOLOGY AND LIFE CYCLE OF *C. PARVUM*

Taxonomically, C. *parvum* belongs to the phylum *Apicomplexa* (having apical complex), class *Sporozoasida* (reproduce by asexual and sexual cycles, with oocysts formation), subclass *Coccidiasina* (life cycle involving merogony, gametogony and sporogony), order *Eucoccidiorida* (schizogony occurs), suborder *Eimeriorina* (independent microgamy and macrogamy develop), family *Cryptosporidiidae* (four naked sporozoites within oocysts) (Levine, 1985). Like other coccidia, C. *parvum* has a monoxenous life cycle which is primarily completed in the gastrointestinal tract of the vertebrates. At the same time, C. *parvum* has many unique characteristics which are quite different from other coccidian. The ability for autoinfection, resistance to antimicrobial agents, the location of the parasites within the host cell membrane etc have set it apart from other coccidian. Previously, it was believed that C. *parvum* lacks in host specificity and can infect a wide variety of mammals. However, recent advances in molecular biology suggest that there are many *Cryptosporidium* species with unique host specificity. C. *parvum* has been reported to infect cattle, humans and ruminants (Caccio et al., 2005). The parasite was first described by Tyzzer in 1907, when he observed them in the gastric mucosa of the laboratory mouse. The first case of human cryptosporidiosis was reported in 1976 and only seven cases were reported before 1982 (Martins and Guerrant, 1995). In 1982, C. *parvum* received increased medical attention when an outbreak of cryptosporidiosis was reported in healthy immunocompetent individuals and when chronic infections were associated with several mortalities in patients infected with human immunodeficiency virus (HIV) who had developed acquired immunodeficiency syndrome (AIDS) (Current et al., 1983). Since then, infections have been associated with mild to severe diarrhea in both immunocompetent and immunocompromised patents throughout the world, specifically in developing countries. In immunocompetent persons, C. *parvum* may cause a short-term (3 to 20 days) diarrheal illness which is usually self limiting. However, in the immunocompromised patient, diarrhea can persist for months and become life-threatening (Current and Garcia, 1991b) .

2.2.1 Life cycle

A simple schematic diagram of the life cycle of *C. parvum* in host cells is provided in figure 2.7. When oocysts are ingested by the host, four sporozoites released in the gut infect the epithelial cells and initiate asexual development.

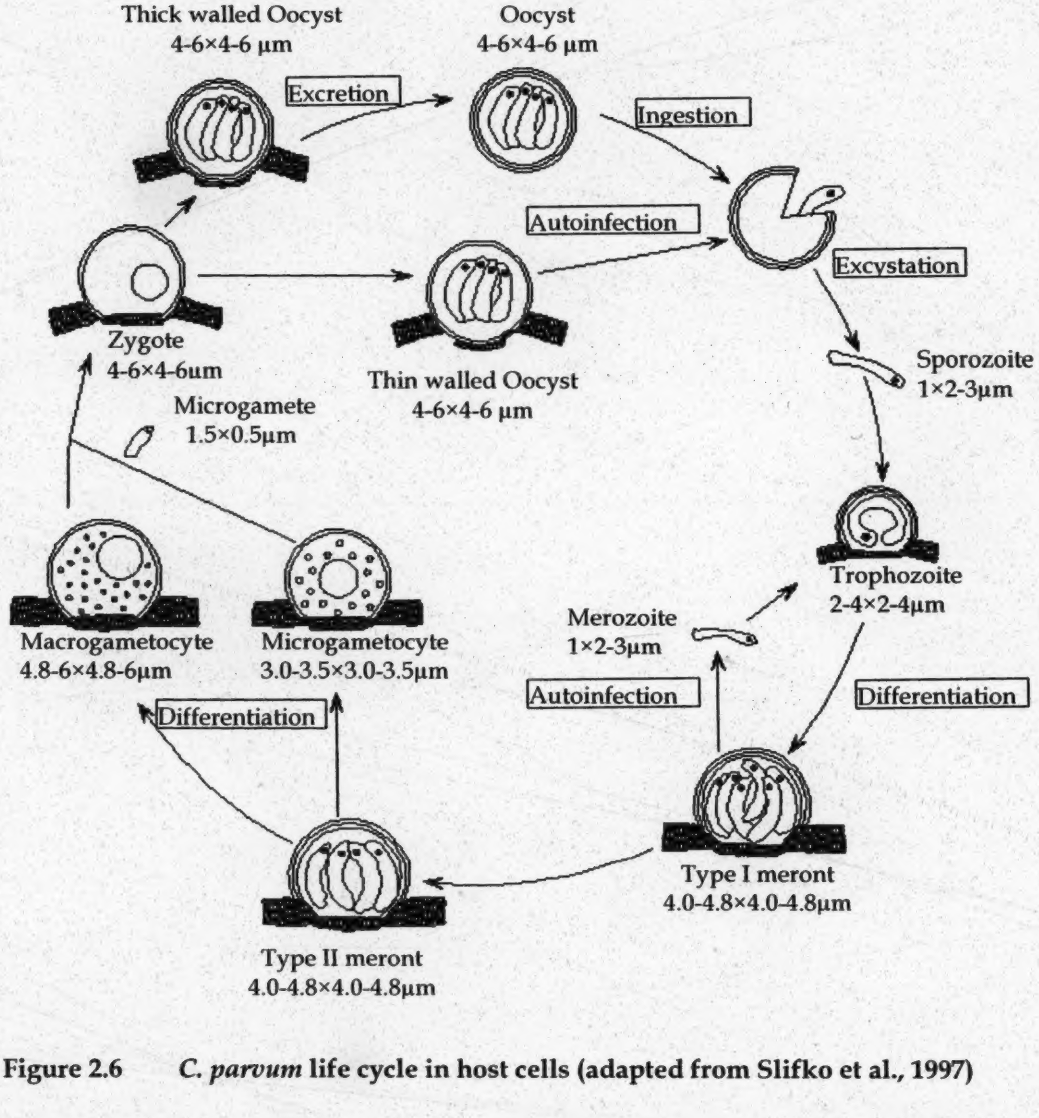

Thick walled Oocyst
4-6×4-6 μm
Excretion
Oocyst
4-6×4-6 μm
Ingestion
Autoinfection
Excystation
Zygote
4-6×4-6um
Thin walled Oocyst
4-6×4-6 μm
Sporozoite
1×2-3μm
Microgamete
1.5×0.5μm
Trophozoite
2-4×2-4μm
Merozoite
1×2-3μm
Macrogametocyte
4.8-6×4.8-6μm
Microgametocyte
3.0-3.5×3.0-3.5μm
Autoinfection
Differentiation
Differentiation
Type I meront
4.0-4.8×4.0-4.8μm
Type II meront
4.0-4.8×4.0-4.8μm

Figure 2.6 ***C. parvum*** **life cycle in host cells (adapted from Slifko et al., 1997)**

C. parvum has monoxenous life cycles where all stages of development (asexual and sexual) occur within one host (Figure 2.7). The infectious stage is the sporulated oocysts which contain four naked, motile sporozoites. Once ingested, the oocyst excysts in the gastro-intestinal tract, releasing the infective sporozoites. Excystation has been reported to be triggered by various factors including reducing conditions, carbon dioxide, temperature, pancreatic enzymes and bile salts (Fayer and Leek, 1984; Reduker and Speer, 1985; Marshall et al., 1997; Smith et al., 2005). The sporozoites escape through a slit-like opening created at one end of the oocyst by dissolution of a special suture in the oocyst wall (Reduker et al., 1985). The released banana-shaped sporozoites attach themselves intimately to the surface of epithelial cells where they invade to initiate the asexual cycle of development. Sporozoites differentiate into spherical trophozoites and nuclear division results in the production of the multinucleated meront stage (merogony). Type I meronts contain 6–8 nuclei that mature into 6–8 merozoites which are then liberated from the tropozoites. Merozoites from type I meronts can either infect neighboring cells, where they undergo another cycle of type I merogony and produce further type I merozoites, or develop into type II meronts. Each maturing type II meronts develops into four type II merozoites which do not undergo further merogony but produce sexual reproductive stages. In sexual multiplication (gametogony), individual merozoites produce either microgamonts or macrogamonts. Microgamonts develop into microgametocytes which produces numerous microgametes. Macrogamonts develop into macrogametocytes which are fertilized by mature microgametes and produce zygotes. The zygotes undergo further asexual development (sporogony) leading to the production of sporulated oocysts containing four sporozoites (Current and Garcia, 1991a; Marshall et al., 1997). Most of the oocyts are thick walled. They are released into the lumen of the intestine and pass out of the body in faeces, where they are infectious for other susceptible hosts. Some oocysts are thin walled and are reported to cause autoinfection by excysting within the same host animal leading to a new cycle of development (Belosevic et al., 2000). The presence of this autoinfection cycle and recycling type I meront result in releasing large number of parasites in the feces. The entire life cycle of the parasite may be completed in as little as 2 days in many hosts and infections may be short-lived or may persist for several months (O' Donoghue, 1995).

2.3 UV INACTIVATION OF *C. PARVUM*

UV inactivation of C. *parvum* has received increased attention recently after it has been shown that C. *parvum* oocysts are highly resistant to chlorine based disinfectants at the concentration and contact times practical for water treatment plants (Korich et al., 1990). Several researchers have evaluated the effect of UV irradiation on the inactivation of C. *parvum*. However, their findings showed considerable variation depending upon the method used to determine the loss in infectivity after UV irradiation. Significant variation was also observed between studies using the same method for the analysis of infectivity. Lorenzolorenzo et al. (1993) used a mouse infectivity method in a bench scale experiment in order to assess the inactivation of C. *parvum* following UV exposure. There is a lack of clarity in the experimental detail and it is difficult to interpret the UV dose delivered based on the information provided in the paper. However, the study achieved 100% C. *parvum* oocysts inactivation when the oocysts were exposed to a UV light source of intensity 1500 mW/sec for >150 min. Following this work, Campbell et al., (1995) examined a *Cryptosporidium* inactivation device (CID) designed for oocysts inactivation in clean water. In the CID, C. *parvum* oocysts were captured in 2 µm filters which were irradiated by LP mercury arc lamps. The investigators demonstrated 2 to 3 log inactivation of oocysts with a UV dose of 8700 mJ/cm^2 using the 4'-6' diamidino-2-phenylindole and propidium iodide (DAPI/PI) vital dye assays and excystation. Clancy et al. (1998) evaluated pulsed UV and conventional LP UV for the inactivation of C. *parvum* in a similar type of CID. Pulsed UV showed 2-log inactivation at a dose of 1900 mJ/cm^2 as evaluated by DAPI/PI and in vitro excystation. Conventional UV was found to be ineffective as determined by the in vitro assay techniques. Mouse infectivity was also performed in the study, but the log inactivation achieved is difficult to interpret from the data provided in the paper. However, both the studies used CID, where the C. *parvum* oocysts were captured in filters and the filters were exposed to UV. This does not represent the actual scenario in water treatment plant where the oocysts are in suspension. Bukhari et al (1999) investigated the inactivation of C. *parvum* oocysts in finished drinking water following MP UV exposure at bench-scale using a collimated beam apparatus and at demonstration-scale using a continuous flow UV reactor. Oocyst viability was assessed using in vitro (DAPI/PI and maximized in vitro exystation) and in vivo (mouse infectivity) methods. Bench-scale results showed that >4.0 log inactivation was achieved at a dose of 41 mJ/cm^2 as measured by mouse infectivity assay. In vitro studies showed very little or no inactivation at this or higher UV doses. The dose delivery in the demonstration scale UV reactor was calculated using a mathematical model and 2-log inactivation was achieved at a dose of 19 mJ/cm^2. The outcome of this study proved that in vitro assays grossly overestimated the oocysts infectivity following UV irradiation compared to the in vivo assay. This is quite understandable as in vitro assays only measure the viability of oocysts, not their infectivity. The UV irradiation damages the DNA of the oocysts, which prevents them from replicating and subsequently from infecting, but these oocysts might be still viable and they might have the ability to excyst in a suitable environment. This explains why past studies found very high dose requirement for oocysts inactivation. Clancy et al. (2000) observed the effect of LP and MP UV irradiation on the inactivation of C. *parvum* as assessed by mouse infectivity. These investigators found that even at low UV doses (3-9 mJ/cm^2), the log inactivation of C. *parvum* oocysts for MP and LP lamps ranged from 3.4 to > 4.0 and 3.4 to 3.7, respectively. The authors also observed the effect of particulate matter on UV inactivation by exposing C. *parvum* oocysts suspended in backwash recycled water (turbidity around 11 NTU) to MP UV light. The results suggested that high level of inactivation (>4.5 log) was achieved at a dose of 3 mJ/cm^2 indicating that the high concentration of particles present in the backwash water did not have any impact on UV inactivation of C. *parvum*. However, the authors concluded that these results should be interpreted with caution as the characteristics of particle association or aggregation of oocysts in spiked backwash water might be different from that in filtered water. Craik et al. (2001) used both LP and MP lamps in a bench scale experiment to evaluate the inactivation of Cryptosporidium oocysts suspended in filtered water and laboratory buffered water. At low dose range, oocysts inactivation increased rapidly showing 2 and 3-log inactivation at dose levels 10 mJ/cm^2 and 25 mJ/cm² respectively. However, tailing effect on the UV dose response curve was observed at higher doses. Low-pressure and medium-pressure lamps did not show any significant difference in inactivation when compared based on germicidal dose. This observation was also supported by Linden et al., (2001) who found that wavelengths ranging from 250 to 275 were most effective for UV inactivation of *Cryptosporidium* parvum and as both MP and LP showed similar germicidal activity at this range of wavelengths, they are equally effective in inactivating *C. parvum* oocysts.

Although, animal infectivity has been considered as the "gold standard" for assessing *C. parvum* infectivity, this technique is costly, tedious and raises ethical concerns. This has persuaded some researchers to look for alternative methods which are reliable and at the same time convenient and cost effective. In vitro cell culture has been proven to be a suitable alternative as it was found to be comparable with mouse infectivity assays (Mofidi et al., 2001; Shin et al., 2001; Rochelle et al., 2002a; Rochelle et al., 2002b; Slifko et al., 2002). Mofidi et al. (2001) used a cell culture-polymerize chain reaction (CC-PCR) method to evaluate the UV susceptibility of *C. parvum* and the researchers achieved 1-log and 2-log inactivation at dose levels of 7.5 and 11 mJ/cm² respectively using both MP and pulsed UV irradiation. Shin et al. (2001) used cell culture-immunofluorescence assay technique and they achieved >3 log removal at a dose level of 3 mJ/cm² when *C. parvum* oocysts were exposed to LP UV irradiation.

DNA repair potential of *C. parvvm* following UV inactivation is another issue which has been examined by several authors (Shin et al., 2001; Morita et al., 2002; Zimmer et al., 2003; Rochelle et al., 2004). Despite the presence of DNA repair genes, no sign of DNA repair (dark or light) has been observed in UV inactivated *C. parvum* oocysts exposed to different dose levels.

Figure 2.8 shows the UV dose response curves of *C. parvum* developed by several researchers and Table 2.1 summarizes the experimental conditions adopted in those studies. Large variation was observed in the log inactivation values obtained in all those studies. These variations can be attributed either to the different experimental conditions adopted in those studies or to the inherent variation that exists in C. *parvum* infectivity assays. From Figure 2.8, it can be observed that at low doses the dose response curves are almost linear but at high doses the curves show significant tailing. The exact reason for this tailing phenomenon is still unknown. However, shielding of UV light caused by particle association or aggregation of oocysts is one of the possible reasons contributing to this tailing effect (Craik et al., 2001).

Table 2-1 Summary of experimental conditions adapted in different studies involved in developing UV dose response relationship for *C. parvum*.

Reference	Exp. type	Assay used	Lamp type	Water quality
(Bukhari et al., 1999)	Cont. flow	Mouse infectivity (CD-1)	MP	Water treatment plant filtered water
(Clancy et al., 2000)	Batch	Mouse Infectivity (CD-1)	MP/LP	Lab DI water/backwash recycle supernatant
(Craik et al., 2001)	Batch	Mouse infectivity (CD-1)	LP/MP	Lab buffered water/water treatment plant filtered water
(Mofidi et al., 2001)	Batch	Cell culture (HCT-8) and RT-PCR	MP/Pulsed UV	Water treatment plant filtered water
(Shin et al., 2001)	Batch	Cell culture (MDCK) and FDM	LP	Lab water
(Rochelle et al., 2004)	Batch	Cell culture (HCT-8) and RT-PCR	LP	Lab water

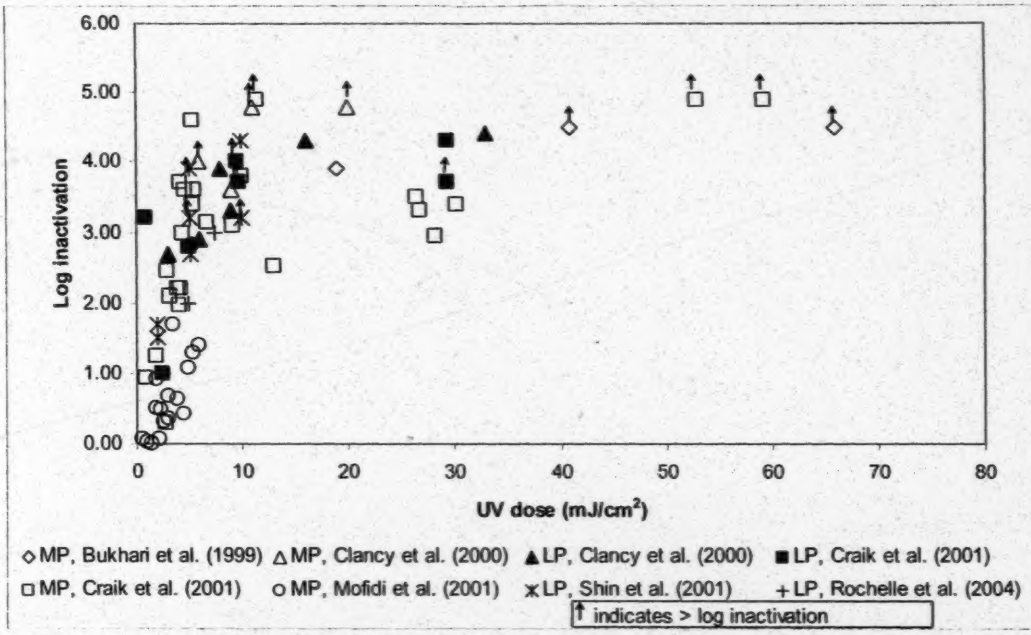


Figure 2.7 **UV dose response curves of *C. parvum* obtained from published peer reviewed studies. The arrows indicate that actual inactivation was greater than the indicated data point (i.e. no infections were observed in either the cell culture wells or in the animals).**

In the UV disinfection guidance manual (USEPA, 2003), published as a part of the LT2SWTR (USEPA, 2006), the USEPA compiled all the published data regarding UV dose response of *C. parvum* in order to develop a standard dose response curve. Only studies where the experimental conditions were clearly documented and where accurate UV dose response calculations were possible were considered during compilation. Only results form studies that used LP lamps on the bench-scale were included in the statistical analysis. MP lamps were not considered because of absence of a standard method for UV dose calculation for the polychromatic MP lamps. Also, only studies that were performed on filtered water, high quality unfiltered water (turbidity less than 1 NTU) or laboratory water were included. A Bayesian statistical analysis of the compiled data set was performed in order to establish a standard UV dose response relationship for *C. parvum*. The final UV dose response curve is based on the Bayesian output of the 90% confidence bound (Cotton and Passantino, 2005). Based on this UV dose response relationship, the USEPA provided a dose table which specifies the UV dose required for utilities to obtain credit for C. *parvum* inactivation. According to that table, a UV dose of 12 mJ/cm^2 is required to achieve 3 log inactivation of C. *parvum* (USEPA, 2003).

2.4 METHODS FOR DETECTING INFECTIVITY OF C. *PARVUM*

Many methods are available for detection of C. *parvum* in drinking water, but they provide little information about the viability or infectivity of the oocysts. Viability is defined as the ability of the parasites to metabolize and reproduce and infectivity is the ability to infect human or animals. Oocysts present in the water have no public health significance if they are not infectious. The current standard protocol for detection of C. *parvum* in water is the USEPA's Method 1622 for *Cryptosporidium* and Method 1623 for *Giardia* and *Cryptosporidium*. Although these methods have been reported to provide good recovery efficiency, they are unable to evaluate the infectivity of the oocysts (LeChevallier et al., 2003). There are several methods available that have been employed by different researchers to determine viability and/or infectivity of the oocyts. Of those, animal infectivity and cell culture have been proven to be most reliable particularly in UV disinfection studies. Animal infectivity study using the model is currently recognized as the "gold" standard for detection of infectious C. *parvum* oocysts. The general procedure of this method includes administering 4-5 day old mice (i.e. CD-1, BALB/c) an intragastic inoculum of C. *parvum* oocysts and examining the intestine of these mice for detecting infection after 7 days. The detection technique includes microscopic analysis of mouse intestine or using flow cytometry in conjunction with fluorescein-labeled anti-*Cryptosporidium* monoclonal antibody (Neumann et al., 2000). However, the mouse infectivity method has several limitations. The use of animals in scientific research raises ethical concerns, and animal-based assays are expensive and time-consuming and have significant hidden costs, such as the maintenance of the animal storage facilities and license fees. Also, there is considerable variation in the C. *parvum* disinfection data generated with mouse infectivity models, although much of the variation may have been due to experimental design rather than a failing of the actual mouse models. A further disadvantage is the inability of mouse infectivity models and other standard animal models to support infection with genotype 1 isolates of C. *parvum* (Widmer et al., 2000) , now referred to as *Cryptosporidium hominus* (Johnson et al., 2005).

2.4.1 Cell Culture

Cell culture techniques have been considered to be a good surrogate for the "gold standard" mouse infectivity model as they are fast, relatively inexpensive and correlate well with the mouse model (Quintero-Betancourt et al., 2002). The USEPA's LT2SWTR recognizes cell culture based methods as valid alternative for animal infectivity and has included the cell culture based UV disinfection studies in developing the standard UV dose criteria for C. *parvum* (USEPA, 2006). The basic principle of this method is to inoculate a host cell line with C. *parvum* oocysts, where they are allowed to grow for a certain period of time. After incubation, the cells are analyzed using microscopic techniques or molecular detection methods in order to detect the life stages of C. *parvum* in host cells. The number of oocysts excysted indicates the infectivity of the oocysts.

Many different cell lines have been reported to support C. *parvum* infection. Infection can be defined as development of one or more stages of C. *parvum* life cycle following inoculation with oocysts or excysted sporozoites. Such stages include trophozoites, meronts, macrogametes and microgametocytes. Complete development of Cryptosporidium has been reported in Caco-2, RL95-2, BFTE, THP-1 and MDBK cells (Rochelle and Leon, 2001). There is currently no consensus on which cell line is the most appropriate for studying in-vitro development of C. *parvum*. Selection of a suitable cell line depends on various factors. These factors include number of life cycle stages developed in the host cells, extent of development, relative ease of detection, etc. Representation of the human intestine is also an important factor in selecting cells. Upton et al. (1994) compared development of C. *parvum* oocysts between Madin-Darby bovine kidney (MDBK) cells and 10 additional host cell lines. Results revealed that the human ileocecal adenocarcinoma (HCT-8) cell line supported nearly twice the number of parasite developmental stages as MDBK cells or any of the other host cell types. Yu et al. (2000) compared four different cell lines (AGS, MDCK, HCT-8 and Caco-2) and they showed that the AGS cell line was most susceptible to C. *parvum*, whereas Caco-2 cell line appeared to be the least susceptible. The authors concluded that since AGS cell line originates from human stomach adenocarcinoma, which has epithelial cell nature, it showed higher number of infection.

Selection of appropriate media is also an important factor for development of C. *parvum* in host cells. Different cell lines require different media formulations for optimal growth. Standard cell culture media, such as RPMI 1640 for HCT-8 cells or DMEM for Caco-2 cells, contain all the essential nutrients for cell growth and are typically supplemented with fetal bovine serum (FBS) at concentrations ranging from 5% to 20% (Rochelle and Leon, 2001). Yu et al. (2000) attempted to determine optimum serum concentration for different cell lines and they reported that the greatest number of infections was reached by using 10% FBS in AGS and HCT-8 cells and by 1% FBS in MDCK cells. There have been conflicting reports on optimum serum concentration. Some investigators also suggested that FBS may contain components that inhibit parasite growth (Rochelle and Leon, 2001).

2.4.2 Detection of Infectious C. *parvum* in cell culture

Two most popular methods for detection of infectious C. *parvum* in cell culture are foci detection method (FDM) and polymerase chain reaction (PCR) method. These two methods are discussed in detail in the following.

Foci Detection Method

The foci detection method (FDM), originally developed by Slifko et al. (1997) involves detection of antibody labeled life stages of infectious C. *parvum* in cell culture. In this method, each life stage of C. *parvum* is designated as a focus. Woods et al. (1995) derived a polyclonal antibody specific to sporozoites and other reproductive stages and structures of C. *parvum*. This is the primary antibody used for the FDM method. This antibody combined with a secondary FITC conjugated antibody, can be viewed under fluorescence microscopy.

The development of C. *parvum* in cell culture largely depends on the incubation time after infection. Slifko et al. (1997) observed the time dependent growth of C. *parvum* in cell culture and they found that a 24 hour incubation after infection resulted in foci to oocysts ratio ranging from 0.5:1 to 1.5:1 whereas after 48 hours the ratio ranged from 9.3:1 to 29.6:1. After 24 hours of infection, autoreinfection stages of the excysted sporozoites start and clusters of reproductive stages in the host monolayer are observed after extended period of incubation (48-72 hours) (Bukhari et al., 2005). Therefore detection after 48 hours increases the sensitivity of the method and is advantageous for presence/absence evaluation. In case of UV disinfection studies, the UV exposed oocysts undergoes excystation and invades the cell monolayers but cannot go through the autoreinfection stages resulting in formation of pinpoint invasion (Bukhari et al., 2005). Clusters of secondary infection are differentiated from invasive pinpoints in order to detect the infectious oocysts following UV exposure.

Polymerized Chain Reaction (PCR)

Cell culture based polymerase chain reaction (PCR) or reverse transcriptase-polymerase chain reaction (RT-PCR) methods for the detection of infectious C. *parvum* in cell lines has been successfully used by several investigators (Rochelle et al., 1996; 1997; Di Giovanni et al., 1999; Rochelle et al., 1999; Rochelle et al., 2002a; Rochelle et al., 2004). PCR or RT-PCR are molecular methods which are based on the amplification of DNA or RNA and often specifically messenger RNA (mRNA) (Wiedenmann et al., 1998; Widmer et al., 1999). The basic steps of these methods are: (i) extraction of DNA/RNA from cell culture, (ii) reverse transcription to a DNA complimentary strand (for RT-PCR), (iii) amplification and (iv) detection of the amplified products. Rochelle et al. (1997) described a RT-PCR based cell culture technique where infections were detected in Caco-2 host cells by C. *parvum* specific RT- PCR of extracted mRNA, targeting the heat shock protein 70 (hsp70) gene. Sensitivity of that method was very high and a single oocyst could be detected. Di Giovanni et al. (1999) evaluated the recovery efficiency of infectious C. *parvum* oocysts from surface and filter backwash water samples using a cell culture-PCR method in combination with immunomegnatic separation (IMS) and the authors found that the cell culture-PCR method agreed well with the immunofluorescence assay. However, the results suggested that the IMS-IFA technique slightly overestimated the recovery efficiency of infectious oocysts compared to the cell culture-PCR assay. Although, the PCR or RT-PCR method is a useful tool for detecting infectious *C. parvum* in cell culture, they have several disadvantages including (i) inefficient extraction of RNA from the oocysts, (ii) interferences in the transcription and amplification steps by unexcysted oocysts or other environmental constituents and (iii) small processed volumes and multiple transfers in the transcription and amplification steps. In UV disinfection studies, the cell culture based PCR method has been reported to underestimate the oorcyst inactivation compared to the mouse infectivity assay (Bukhari and LeChevallier, 2003). When UV inactivated oocysts are inoculated in cell culture, they might excyst and invade the cell lines but cannot go through the multiplication and autoreinfection stages. PCR and RT-PCR techniques may not discriminate between UV treated oocysts that excysted and invaded the cell line from those oocysts that actively multiplied within the cell line following invasion, resulting in false positive detection of the infectious oocysts. However, targeting mRNA for detection of infectious oocysts may reduce the risk of false positive detection considering that mRNA degrades rapidly if the oocysts are incapable of causing infection (Rochelle and Leon, 2001). Cell culture-FDM method involves detection clusters of foci which are formed by the oocysts which went through autoinfection in the secondary infection stages and therefore it is less likely to produce false positive detection (Bukhari et al., 2005).

2.4.3 Comparison between Mouse infectivity and cell culture

Slifko et al. (2002) compared the infectivity of *C. parvum* oocysts in cell culture with the mouse model. The FDM method was used to detect clusters of foci in HCT-8 cell monolayer and the wells were assessed for positive/negative infection. A MPN approach was used to quantify the number of infectious oocysts based on the proportions of wells infected following cell culture. *C. parvum* oocysts were exposed to low pressure UV irradiation with doses ranging from 3 to 33 mJ/cm^2. For UV treated oocysts, the correlation between log inactivation determined by mouse infectivity and cell culture was statistically significant ($r^2 = 0.9363$; $p < 0.0001$). Logistic dose response model for both mice and HCT-8 cells were developed. No significant difference at the 95% confidence level ($p = 0.0997$) was observed between the slopes of logistic dose response model for cells and mice. However, the ID_{50} of the mouse models (64 for BALB-c and 119 for CD-1) was an order of magnitude higher than that of the tissue culture model (8) suggesting that the cell culture method is more sensitive compared to the mouse infectivity assay. Shin et al. (2001) also demonstrated that the UV inactivation of C. *parvum* oocysts determined by cell culture-FDM method was not significantly different (at the 95% confidence level) from that determined by the mouse infectivity assay.

Rochelle et al. (2002) developed a logistic dose response model for C. *parvum* using cell culture based RT-PCR assay and compared with the dose response model of CD-1 mice. These authors found that the dose response curves for different cell lines agreed with the dose response curve for CD-1 mice ($R^2 = 0.67$-0.77). Significant correlation ($r = 0.89$, $n = 29$) was also observed between infectivity in HCT-8 cells and infectivity in CD-1 mice for oocysts that had been exposed to UV irradiation. However, the UV doses used in this study were low (ranging from 2 to 8 mJ/cm^2) and the information regarding size of the inoculums and the log inactivation achieved at different doses was inadequate. Further studies are required to compare the cell culture RT-PCR assay with mouse infectivity for oocysts that have been exposed to higher UV doses.

2.4.4 Limitations of cell culture method

The limitations of the cell culture method for detecting infectious C. *parvum* oocysts are as follows:

- A standard method for the cell culture assay is yet to be developed. Researchers use a variety of techniques including different cell lines, culture medium etc.
- The oocyst pretreatment step prior to the infection in cell lines is a concern because this pretreatment may have synergistic effect in disinfection studies
- The age and number of passages of a cell line greatly affect its susceptibility to the parasite.

- C. *parvum* induces apoptosis in cells leading to sloughing of the cells from the surface of the culture plates
- Temperature, storage time after purification, processing, handling and storage of oocysts may affect the ability of the parasites to excyst in cell culture.

2.5 EFFECT OF PARTICLES ON UV DISINFECTION

Suspended and dissolved particles present in filtered and unfiltered water may have a negative impact on the UV disinfection process. Suspended particles can scatter, absorb or block UV light (Qualls et al., 1983). UV light absorbed by the particles is no longer available for disinfection. Scattered light is still available for disinfection, but as the path length of the light traveling through liquid medium increases, the UV energy decreases (Christensen and Linden, 2003). Suspended particles can block light by shading and shielding microorganisms. At the same time, microorganisms can form aggregates with particles or other microorganisms which may prevent the UV light from reaching the microorganism and inactivating them (USEPA, 1999). To date, research examining the effect of particles in drinking water applications on UV disinfection has been limited. However, several studies have been performed using wastewater matrices. Most of these studies used coliform bacteria for assessing the effect of wastewater particulate matter on UV inactivation (Qualls et al., 1983; Qualls et al., 1985; Parker and Darby, 1995; Emerick et al., 1999; Loge et al., 1999; Emerick et al., 2000; Jolis et al., 2001). Qualls et al. (1983) showed that large particles present in a sample of secondary effluent can harbor coliforms from UV exposure and may cause tailing of the inactivation curve at higher doses. These authors passed secondary effluent through 70 μm and 8 μm filters and constructed dose-inactivation curves for the unfiltered and filtered samples. It was observed that the inactivation curve for the 8 μm filtered sample was almost linear and the inactivation was beyond detectable after 4.5 log unit. On the other hand, the survival curves for unfiltered and 70 μm filtered samples showed tailing after 2 to 3 log inactivation units, although the tailing characteristics of the 70 μm filtered samples was less significant than that of the unfiltered samples. The effect of mixed media filtration in a pilot scale UV disinfection system was also evaluated in that study. Results suggested that UV inactivation of coliform bacteria was much better in the filtered water compared to the unfiltered water. Qualls et al. (1985) found that the number of coliforms that survived in different unfiltered secondary effluent samples at a UV dose of 26 mJ/cm^2 was roughly correlated ($p<0.0001$) with the number of suspended particles greater than 40 μm in diameter. Emerick et al. (1999) evaluated the UV inactivation of particle associated coliform bacteria in eight different types of wastewater samples and they found that the number of particles with associated coliform bacteria was directly correlated to residual coliform bacteria concentration (the survivors) after exposure to high UV doses. The authors also concluded that chemically induced flocs provided better protection of the microorganisms from UV light compared to biological flocs. Jolis et al. (2001) found that suspended particles larger than 7 μm had a significant effect on the UV inactivation of coliform bacteria in secondary effluent. To summarize, all these studies indicated that wastewater particles larger than 10 μm have the potential to shield coliform bacteria from being inactivated by UV light.

Research conducted on wastewater provides an indication of effects of aggregation on UV disinfection in drinking water supplies. However, there is a difference in characteristics between wastewater particles and the particles that passed through conventional water treatment processes. Therefore, the particles present in drinking water are likely to have different effects on the UV disinfection system. Several authors have investigated the effect of water quality on UV inactivation (Christensen and Linden, 2003; Batch et al., 2004; Passantino et al., 2004; Amoah et al., 2005) using turbidity as a representative parameter of water quality and they found contrasting results. Passantino et al. (2004) reported that UV inactivation of MS2 bacteriophage in artificial water with turbidity up to 12 NTU and algal content up to 42,000 cells/mL was not significantly different (at the 95% confidence level) than that in DI water. However, the authors used montmorillonite clay to create artificial turbidity and clay particles have been reported to scatter UV light. As this scattering was not taken into account while measuring the absorbance of water, it might have resulted in underestimating the actual UV dose delivered. When UV inactivation of MS2 bacteriophage in natural water was compared with that in DI water, the difference was found to be statistically significant (at the 95% confidence level). This indicates that the particle association of the microorganisms might be different in natural water relative to artificial water. Christensen and Linden (2003) reported that the impact of particles in UV dose delivery in natural water with turbidity up to 10 NTU was negligible as long as UV absorbance of the water was taken into account while determining dose. However, the authors suggested that UV absorbance of water having turbidity >3 NTU should be measured using the integrated sphere method, which accounts for the light scattering of suspended particles. Batch et al. (2004) found that turbidity and particles count did not affect the inactivation of MS2 coliphage in filtered water if the UV absorbance of water was factored into the bench scale UV dose measurements. On the other hand, Amoah et al. (2005) found that particulate matter present in natural water resulted in statistically significant but not very large reduction of UV inactivation of *C. parvum* and *Giardia muris* at turbidity >10 NTU. The authors used mouse infectivity assay for determining the log inactivation of the parasites.

2.5.1 Effect of upstream treatment processes on UV inactivation of *C. parvum*

In a multiple barrier water treatment process, raw water has to pass through coagulation, flocculation, sedimentation and filtration processes prior to UV disinfection. Theses processes play an important role in promoting the aggregation of microorganisms in filtered water and hence the performance of these processes might affect the performance of UV disinfection. The majority of the data on the performance of treatment processes in removing *Cryptosporidium* oocysts and *Giardia* cysts from drinking water have been obtained from pilot-tests, with a few studies performed in full-scale conventional water treatment plants. These studies have demonstrated that protozoan cysts or oocysts removal throughout all stages of the conventional treatment is largely influenced by the effectiveness of coagulation pretreatment. In most of the studies, suboptimal coagulation resulted in significant reduction of the log removal of oocysts in filtered water.

Coagulation is a process which helps to increase the tendency of small particles in aqueous suspension to aggregate and form large settelable flocs. The degree and mechanism of coagulation depends on the type of coagulant added, dose, size, shape, surface characteristics of the particles, and the composition of source water. C. *parvum* oocysts are spherical in shape with a surface composed of a complex matrix of glycoproteins (Karaman et al., 1999). Oocysts have a negative surface charge under typical environmental conditions (Drozd and Schwartzbrod, 1996; Ongerth and Pecoraro, 1996; Karaman et al., 1999), likely due to the presence of carboxylate, carboxylic, and phosphate groups on the oocyst surface (Karaman et al., 1999). Both steric and electrostatic forces can play a role in oocyst aggregation. It has been hypothesized that proteins can extend from the oocyst surface due to charge repulsion between ionizable surface groups, thus giving the oocyst surface a brushlike conformation (Considine et al., 2002). This may give rise to steric forces that promote oocyst stabilization. In addition, electrostatic repulsive forces between negatively charged oocysts and suspended sediments, which are also typically negatively charged, may hinder the aggregation of oocysts. However, electrostatic interactions are highly dependent on both solution and surface chemical conditions, so oocyst aggregation can be favored under specific solution chemical conditions or with particular types of particles (Searcy et al., 2005). Conventional coagulant and coagulant aids can promote the binding of C. *parvum* oocysts with other particles which may result in complete encapsulation into large flocs. The mechanism of oocysts removal by coagulation and flocculation is still not well understood. Precipitate enmeshment is considered the optimal mechanism of coagulation and flocculation for removal of protozoan cysts in water treatment systems (Betancourt and Rose, 2004). Xagoraraki and Harrington (2004) evaluated the ability of alum coagulation to remove C. *parvum* oocysts suspended in a solution containing natural organic matter (NOM) and the authors found that the C. *parvum* oocysts did not bind with other particles through the charge neutralization mechanism. NOM played a key role in removing the oocysts by acting as a bridging agent between the surface of the oocysts and the surface of the aluminum hydroxide floc particles. However, the ability of the oocysts to aggregate with other particles or oocysts depends on the zeta potential of the oocyst surface which in turn depends on the purification and storage of the oocysts in the laboratory (Butkus et al., 2003). Therefore, the mechanism of particle association or aggregation of oocysts through coagulation and flocculation in natural water might be completely different from that observed in the bench scale experiment.

Filtration plays a significant role in removing *C. parvum* from drinking water. LeChevallier et al. (1991) examined 66 conventional water systems in the USA and found that most of the utilities achieved 2-2.5 log removal of *Giardia* cysts and *Cryptosporidium* oocysts by clarification and filtration. The investigation revealed that the production of low turbidity water (<0.5 NTU) did not ensure cysts or oocyst free effluents. Nieminski and Ongerth (1995) conducted a study on removal of *Giardia* and Cryptosporidium by filtration at a full-scale treatment plant and a pilot plant operating under conventional treatment and direct filtration regimes. They confirmed the necessity of attaining low filtered water turbidity and the importance of maintaining optimum coagulation and flocculation. They concluded that a properly operated treatment plant producing finished water turbidity of 0.1 to 0.2 NTU, using either the direct filtration mode or the conventional treatment mode, could achieve 3-log removal of *Giardia* cysts and about 2.6-log removal of *Cryptosporidium*. In a pilot scale experiment using multimedia filtration following coagulation and flocculation, Ongerth and Pecoraro (1995) found 3 log removal of *Cryptosporidium* oocysts and slightly higher than 3 log removal of *Giardia* cysts when optimal coagulation was maintained and filtered water turbidity was low. However, when coagulation was suboptimal, the oocysts and cysts removal decreased to 1.5 logs and 1.3 logs respectively.

Turbidity and particle counts are reliable indicators for overall treatment performance but they are not reliable quantitative surrogates for *Cryptosporidium* removal (Lechevallier and Norton, 1992; Nieminski and Ongerth, 1995). In a pilot plant study conducted by Huck and colleagues (2002), there was a 2-log difference in *Cryptosporidium* removals between two pilot plants operated at optimal condition and that produced similar low turbidity (<0.1 NTU) filtered water. When the coagulation was suboptimal, log removal decreased by several log units (>2.0) even at turbidity levels <0.3 NTU. It was also observed that at early stages of breakthrough, *Cryptosporidium* removal decreased substantially even though there was no remarkable increase in turbidity. In another laboratory study, suboptimal coagulation had a significant effect on removal of *Cryptosporidium* in both settled water and filtered water (Dugan et al., 2001). Glasgow and Wheatley (2001) showed that particles with size range 3-7 μm can exhibit breakthrough without any significant change in turbidity or removal of smaller size particles. Harrington et al. (2003) tested the effect of filtration condition on removal of different microorganisms (*C. parvum, Encephalitozoon intestinalis, E. coli, A. hyydrophila* and bacteriophage MS2) and found that turbidity breakthrough was accompanied by the breakthrough of all the microorganisms.

2.6 NEED FOR THIS RESEARCH

Until now, most of the UV dose-response studies of *C. parvum* have been based on oocysts suspended in relatively clean water. The general procedure is to suspend oocysts in laboratory water or filtered water and then expose to UV irradiation. The dose table established by USEPA in the LT2SWTR (USEPA, 2006) for reactor validation was developed based on the studies where the microorganisms were spiked into laboratory water or filtered water during UV exposure. Particle association or aggregation of the oocysts was not taken into account while determining the UV dose response. But the actual scenario in a water treatment plant is completely different. In a multi barrier water treatment plant, the oocysts have to pass through several water treatment processes prior to UV disinfection. These processes (i.e. coagulation, flocculation, settling and filtration) actually promote the particle association or the aggregation of the oocysts and as a result the UV energy reaching the target microorganisms might be reduced or completely blocked. Therefore, the dose response curve generated for pure suspension of oocysts might not be able to estimate the UV dose required for attaining a certain level of inactivation in filtered water.

Several studies have tried to evaluate the effect of particles on UV disinfection of *C. parvum* by spiking the oocysts in a suspension of elevated particle concentration. The water quality is usually measured by the turbidity. This method has several setbacks. Firstly, the artificial particles used to elevate the particle concentration may have different light scattering properties compared to the particles present in natural water. Secondly, as both suspended particles and oocysts present in a spiked suspension have negative surface charge; they are unlikely to associate with each other under normal condition. Thirdly, turbidity measurement works on the principle of light scatter from suspended particles, but the technique does not provide details of the size, size distribution, composition and structure of the particles (Glasgow and Wheatley, 2001), hence it is not a reliable indicator of the level of the oocyst aggregation in filtered water .

Very few studies have attempted to simulate the water treatment processes in order to observe their effect on the UV disinfection. Conventional water treatment processes (coagulation, flocculation, sedimentation and filtration) have the potential to encourage particle association of the microorganisms. The floc particles can completely engulf the microorganisms and prevent UV light penetration. Templeton et al. (2005) observed the effect of coagulation and flocculation on particle association and UV inactivation of viral surrogates (MS2 coliphage and bacteriophage T4). A synthetic mixture of water containing three different types of particles (Kaolin clay, humic acid powder and activated sludge) were spiked with the viral surrogates and coagulated at optimum condition. The coagulated water was then exposed to UV at different dose levels. Results showed that although a large percentage of the viral surrogates were attached to the kaolin clay particles, the log inactivation of the microorganisms did not decrease significantly relative to the control (pure suspension of the phages in buffered water). On the other hand, the attachment of the microorganisms to humic acid and activated sludge floc particles resulted in statistically significant reduction of the UV inactivation. The authors concluded that, since clay particles do not absorb UV light heavily relative to humic acid and activated sludge particles and since the microorganisms were only attached to the surfaces of the clay particles, they did not provide any protection to the microorganisms from UV light. Although, this study provides a good insight into the effect of coagulation and flocculation on UV inactivation of the microorganisms, it is not representative of actual water treatment plant scenario. Natural water matrices contain a composition of different types of particles which may behave differently from the particles in synthetic water. Furthermore, although coagulation and flocculation promotes aggregation, the flocs are relatively weak and feathery, which might not prevent the penetration of UV light. If the settled water is passed through the interstices of filter media, the flocs are compacted and shaved off. This may produce a floc that results in complete blockage of UV light.

Uvbiama and Craik (2005) conducted a study where the investigators simulated upstream water treatment processes at the bench-scale and observed how these processes affected the UV inactivation of *Bacillus subtilis* in filtered and backwash water. The researchers introduced a homogenizing step to break up the aggregated spores either before or after UV exposure. Homogenized and non-homogenized samples were exposed to different UV doses and dose response curves were constructed and compared. Results suggested that the difference in UV inactivation rate of *B. subtilis* between filtered water and suspension of pure spores was statistically significant at the 95% confidence level. The difference was even greater when backwash water was compared with pure spore suspension. More importantly, UV inactivation of the spores was greater when the samples were homogenized before UV exposure compared to samples that were homogenized after UV exposure. Suboptimal coagulation was used in this study in order to represent worst case scenario and also to recover sufficient spores for determining higher levels of log inactivation following UV disinfection. The findings of this study indicate that the upstream treatment processes such as coagulation, flocculation, settling and filtration might encourage particle association and aggregation of microorganisms in filtered water which in turn can prevent UV light from reaching the target microorganisms and inactivating them. The outcome of this study suggests that there is a possibility that a similar effect might exist in case of UV inactivation of *C. parvum* in filtered water. However, *C. parvum* oocysts (3 to 6 μm) are larger than *B. subtilis* spores (1 to 2 μm) and therefore the characteristics of oocyst aggregation in filtered water might be different from that of spore aggregation. In addition, experiments involving live *C. parvum* oocysts are substantially more complex and difficult than *B. subtilis* experiments.

The research study described in this thesis is similar to the study conducted by Uvbiama and Craik (2005) except that C. *parvum* was used as a target microorganism because of the public health significance. This is the only study where live C. *parvum* oocyts were passed through simulated conventional water treatment processes and the effect of these treatment processes on UV inactivation of the oocysts was evaluated using a cell culture infectivity assay. Suboptimal coagulation was used intentionally to produce filtered water with high turbidity and to recover sufficient oocysts for determination of log inactivation following UV exposure. Since most of the waterborne outbreaks were associated with turbidity breakthrough following suboptimal coagulation, this study provided an opportunity to observe the characteristics of aggregated C. *parvum* oocyts during turbidity excursions and subsequently the effect on UV inactivation. In addition, direct measurements and qualitative examination of the oocyst aggregation provided useful information regarding the possible types of aggregation in filtered water.

3.0 EXPERIMENTAL MATERIALS AND METHODS

This chapter contains a detailed description of the experimental methods and materials used in this study. The topics discussed here include raw water sample collection, simulation of water treatment processes, UV exposure experiments, *C. parvum* oocyts production, in vitro cell culture, infectivity analysis using logistic dose-response model, impact of homogenization on the infectivity of *C. parvum* oocyst, direct measurement of oocyst aggregation and microscopic examination of oocyst aggregation.

3.1 RAW WATER SAMPLE COLLECTION

North Saskatchewan River water samples were collected in 20 L plastic containers from the Rossdale water treatment plant quality control laboratory in Edmonton, AB. Prior to the collection, the containers were soaked into 30% bleach overnight and washed thoroughly at least 3 times. After collecting, the raw water samples were stored at 4°C until their use in the experiments. Raw water samples were collected twice during the course of the study. Table 3.1 shows the water quality characteristics of the raw water samples,

Table 3-1 Water quality characteristics of the raw water samples collected in July, 2005 and November, 2005.

Water parameter	July (2005) sample	November (2005) sample
Turbidity, NTU	43.5	5.3
pH	8.3	7.75
Alkalinity, mg/L	134	136
Total Hardness	176	178
Conductivity, μS/cm	356	339
Color, TCU	13	N/A

N/A – Not available

3.2 WATER TREATMENT PROCESS SIMULATION

One of the main objectives of the study was to determine the UV inactivation of *C. parvum* oocysts that break through a granular media filter in a simulated water treatment process. Two major technical difficulties had to be addressed before designing the experiments. Firstly, several studies have reported good oocysts removal efficiency of the dual sand-anthracite media filters under optimal coagulation/flocculation conditions (Emelko et al., 2005). Since the present study involved evaluation of UV inactivation of the oocysts, a significant number of oocysts were required for the infectivity analysis in order to determine the maximum log inactivation achieved at a particular UV dose. For example, based on past research conducted on the UV inactivation of *C. parvum*, 2 to >3 log inactivation can be achieved at UV dose of 5 mJ/cm^2. Therefore, in order to have the freedom to calculate up to 3 log inactivation following UV exposure, it was necessary to recover approximately 3×10^6 oocysts from the filtered water for the infectivity assay. This is based on an ID_{50} of 50 oocysts and inoculation of 8 wells. A conventional granular media filtration process can be expected to remove 3 log of oocysts in the raw water if the coagulant dose and conditions have been optimized (Emelko et al., 2005). Assuming approximately 3 log removal of oocysts during filtration, approximately 3×10^9 oocysts would need to be seeded into the raw in order to ensure the necessary oocyst recovery in the filtered water. It is difficult as well as very expensive to produce such a large number of oocysts. Therefore, sub-optimal coagulation was intentionally adopted in this study to ensure that enough oocysts were available in the filtered water to permit meaningful determinations of oocyst infectivity reduction. Since most of the water borne outbreaks of *C. parvum* were reported to be associated with sub-optimal filter operation (Mackenzie et al., 1994; PHAC, 2001), it also provided an opportunity to observe the level of oocysts aggregation that break through the treatment barrier during periods of sub-optimal operation and subsequently the effect on UV inactivation. Secondly, for evaluating the effect of particle association or oocysts aggregation on UV inactivation, the filtered water had to be exposed to UV irradiation and since collimated beam apparatus was used for UV exposure, the volume of filtered water required to be irradiated had to be kept small in order to minimize the total number of exposures. Concentration by centrifugation, membrane filtration or any other means of reducing the filtrate volume was purposefully avoided because these techniques could potentially alter the state of aggregation of the oocysts. To solve this problem a small amount of raw water (i.e. 2L) was seeded with large number of oocysts (i.e. 5×10^8) and coagulated/flocculated/settled along with unseeded raw water. The seeded settled water was then spiked into the filter influent during stable filter operation and collected after filtration. To optimize the oocysts recovery in the filter effluent, the collection time of the seeded filtrate was determined from a tracer test and trial filtration runs with formalin fixed oocysts.

At this point, some of the terminologies which will be used throughout the thesis require explanation. The terminologies with their respective explanations are provided in the following:

- A "filtration run" indicates a simulated water treatment process run which includes coagulation, flocculation settling and filtration.
- The term "seeded/settled water" refers to raw river water that had been seeded with live or formalin fixed C. *parvum* oocysts and was collected after the coagulation/flocculation/settling process.
- The term "unseeded/settled water" refers to water collected after coagulation/flocculation/settling process but which was not seeded with oocysts.
- The term "seeded/filtered water" refers to the filter effluent which was collected following filtration of "Seeded/settled water".
- The term "Unseeded/filtered water" is the filter effluent that was collected following filtration of "unseeded/settled water".

3.2.1 Coagulation, flocculation and settling

A jar testing apparatus (PB-700 Jar tester, Phipps and Bird, Richmond, VA) was used to simulate the coagulation, flocculation and settling process at bench scale (Figure 3.1). The jar testing protocol used in this study was similar to that used by EPCOR Water Services at Rossdale water treatment plant. Three jars (2L B-kars) were filled with 2L of raw river water. One jar was seeded with live or formalin fixed C. *parvum* oocysts previously produced from Holstein calves. The number of oocysts seeded into the raw water was dependent on the maximum log inactivation predicted at each UV dose level. For UV dose levels of 5 and 40 mJ/cm^2, approximately 5×10^8 and 3×10^9 oocysts were seeded in the raw water, respectively. These numbers were selected based on trial runs with formalin fixed oocysts which ensured the availability of enough oocysts in the filtered water for calculation of the log inactivation predicted at each dose level. The content of each jar was initially mixed at moderate speed for about 10 minutes. Mixing was increased to >300 rpm and alum (Cleartech, Edmonton, AB) was added. After 1 min, the speed was reduced to 75 rpm and Percol LT27A anionic polymer (Allied Colloids Inc., Brampton, ON) was added to each jar. After 3 min, the speed was reduced to 50 rpm for 3 min followed by another reduction in speed to 25 rpm. After 3 min at 25 rpm, the water in each jar was allowed to settle for an additional 3 min. Then the supernatant of the unseeded and seeded settled water was collected in separate containers (4L conical flasks). The average turbidity of the settled water was measured using a Model 965 Turbidimeter (Orbeco Hellige Turbidimeters, Orbeco Analytical Systems, Farmingdale, ON). The alum and polymer doses used during coagulation of different filtration runs are shown in Table 3.2. Again, these doses were selected based on trial filtration runs with an objective to recover sufficient oocysts in filtered water required for UV exposure experiments.

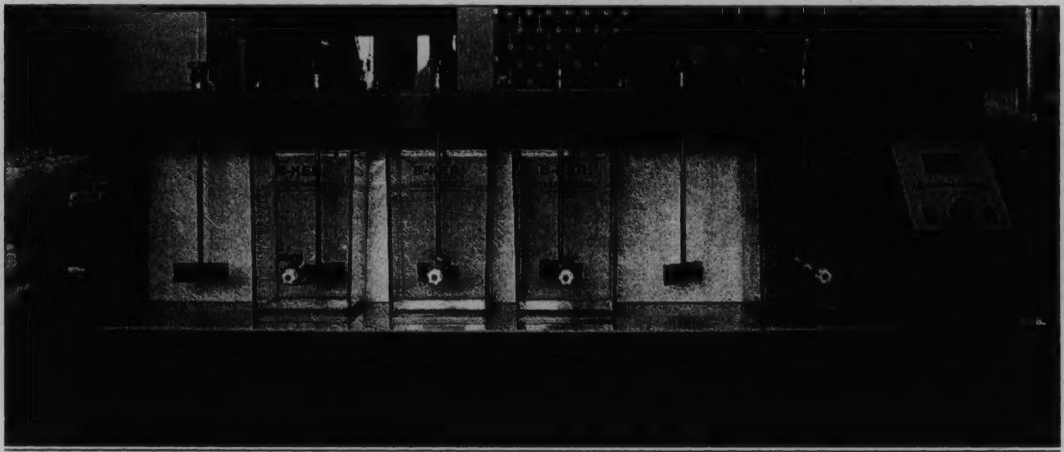


Figure 3.1 **Jar testing apparatus used for simulation of coagulation, flocculation and settling**

Table 3-2 **Alum and polymer dose used for coagulation in different filtration runs**

Filtration run no	Alum dose	Polymer dose
1,2	10 mg/L	1 mg/L
3, 4, 5 , 6	5 mg/L	0.5 mg/L

3.2.2 Filtration

The filtration procedure used in this study was originally adopted from Uvbiama and Craik (2005). Slight modifications were applied in order to address the biohazard issue associated with filtering water containing live *C. parvum* oocysts. Figure 3.1 shows a schematic diagram of the bench scale filtration process. The filter column consisted of a 100 cm long tube (2.54 cm internal dia) and an overhead tank (10.16 cm internal dia) constructed with clear PVC material. The tube was filled with 20 cm of crushed quartzite sand and 7.3 cm of anthracite filter grade coal (AWI filters, Calgary, AB). This is the same media that is used by EPCOR water services at Rossdale water treatment plant. The height of the sand and anthracite filter media was calculated based on ratio of the height of these media at Rossadale water treatment plant (55cm of sand and 20 cm of anthracite coal). Table 3.3 provides the physical properties of the sand and anthracite coal.

Prior to filtration, the filter column was backwashed with approximately 10L of DI water in order to remove silt and dust from the filter media. The filter media was allowed to settle for 30 min and the flow of unseeded/settled water through the filter column was started. After filtering the unseeded/settled water for ~30 min, the entire volume of the seeded/settled water was filtered through the column. The filtration of unseeded/settled water was started again and was continued for an additional 30 min. The seeded/filtered water was collected separately and stored for future experiments. The collection time of seeded/filtered water was optimized based on a tracer test and trial filtration runs with formalin fixed oocysts. The objective was to capture as many oocysts as possible keeping the volume of seeded /filtered wateras small as possible. The details of the tracer test conducted on the filter column are provided in Appendix B.

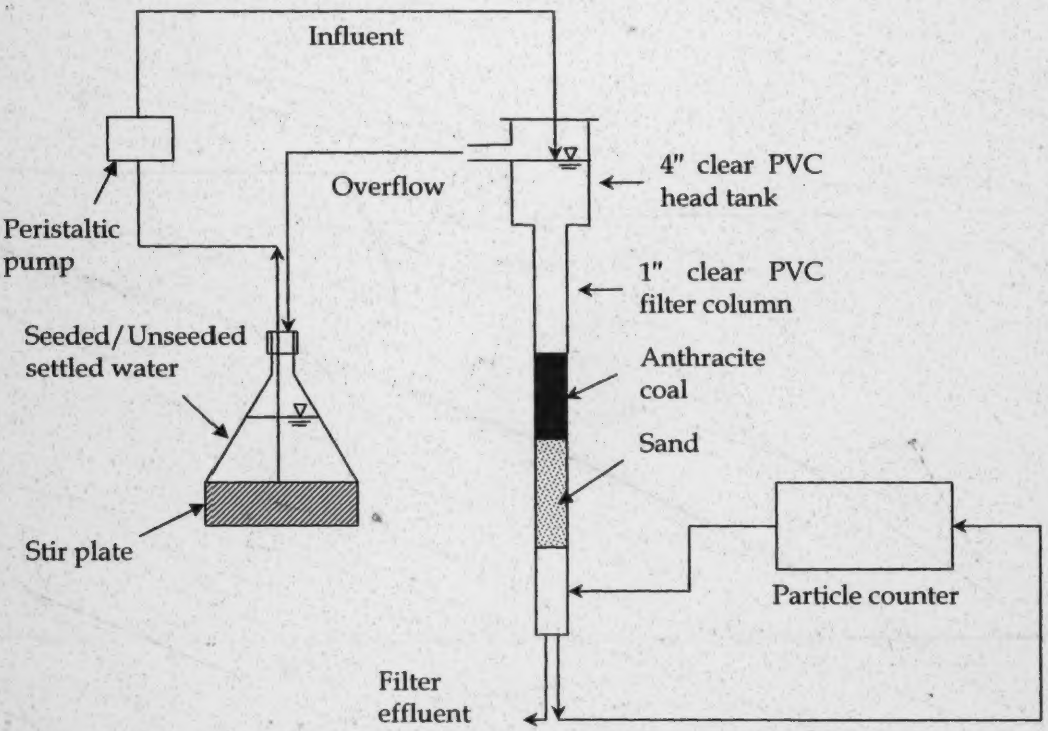

Influent
Overflow
4" clear PVC head tank
Peristaltic pump
1" clear PVC filter column
Seeded/Unseeded settled water
Anthracite coal
Sand
Stir plate
Particle counter
Filter effluent

Figure 3.2 **Schematic diagram of the simulated filtration process**

The filter was designed and operated in a constant head declining rate mode. The overflow from the head tank was returned to the conical flask containing the settled water. The settled water was stirred continuously but gently in order to keep the particles suspended. The typical flow rates at different points during filtration are shown in Table 3.4. During the filtration run, sub-samples were collected in order to measure turbidity at regular intervals. An online particle counter (L & H Potable Water Sensor, L & H Environmental Inc. Roseberg, OR) was used to monitor the particle size distribution and concentration. The particle counter measured particles in ranges ≥ 2, ≥ 5, ≥ 15, ≥ 25, ≥ 50 and ≥ 100 μm size. The purpose of measuring turbidity and particle concentration was to monitor the filter effluent quality and also to find out whether these parameters can indicate oocysts breakthrough in the filter effluent. During the period of the study, six filtration runs were conducted. A summary of the filtration runs and their respective use is provided in Table 3.5

Table 3-3 Physical properties of the filter media

Property	Sand	Anthracite Coal
Bulk density	1362 kg/m³ (dry basis)	N/A
Color	Crystal White	Black
Sphericity	< 0.6 (highly angular)	N/A
Moh Hardness	7	N/A
Acid Solubility	Nil	N/A
Specific Gravity	2.60 - 2.65	1.4 - 1.6 ± 0.05
Effective size	0.35 mm	0.8 - 0.9 mm
Uniformity Coefficient	< 1.5	N/A

N/A - Not Available

Table 3-4 Typical flow rates at different points of the filtration run

Point	Flow rate (m/h)
Inflow	20.7
Overflow	13.6
Filtrate	6.4
Particle counter	7.1

Table 3-5 Experimental conditions of different filtration runs

Filtration run No.	Raw water sample collection date	Type of oocyts seeded	Purpose
1	July, 2005	Formalin fixed	Serial filtration and microscopy
2	July, 2005	Formalin fixed	Serial filtration and microscopy
3	November, 2005	live	UV experiment (5 mJ/cm²)
4	November, 2005	live	UV experiment (5 mJ/cm²)
5	November, 2005	live	UV experiment (40 mJ/cm²)
6	November, 2005	Formalin fixed	Serial filtration and microscopy

3.3 UV EXPOSURE EXPERIMENTS

Two sets of UV exposure experiments were conducted during the course of the study. For the first set, C. *parvum* oocysts suspended in 0.053M phosphate buffered water were exposed to UV and for the second set, oocysts that passed through a bench scale water treatment process and collected in filtered water were exposed to UV.

3.3.1 Collimated beam apparatus

UV exposure experiments were carried out using a collimated beam apparatus (Calgon Carbon Corporation, USA). A 10 W low pressure mercury arc lamp (Ster-L-Ray Germicidal Lamp, model G12T6L, 15114, Atlantic Ultraviolet Corporation, Hawpange, NY) housed in the collimated beam unit was used for all the UV exposure trials. The unit uses a pneumatically driven shutter which can be opened and closed to allow the UV light to irradiate the test suspension under controlled environment. The time required for delivering the required UV dose was keyed in to a timing device which automatically controlled the opening and closing of the shutter. A collimation tube (24 cm long) was used to make sure that the UV rays reaching the test suspension were reasonably parallel and uniform. The distance from the lamp to the surface of the test suspension for all the UV exposure trials ranged from 41 to 51 cm. The test suspension was placed on an adjustable platform mounted over a stirrer.

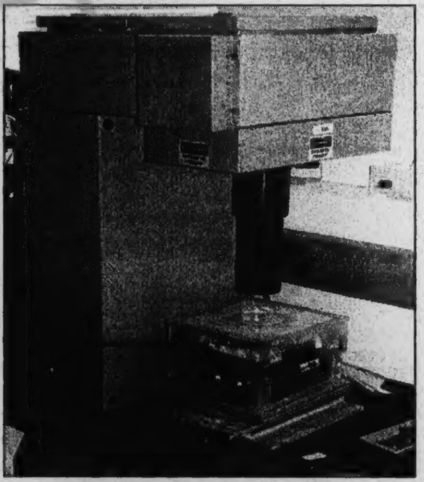


Figure 3.3 Collimated beam apparatus used for UV exposure experiments

3.3.2 Phosphate buffered water

Ooocyst stock suspensions were counted in replicate using a hemacytometer. Oocyst suspensions containing 10×10^6 oocysts in 1 mL of milli-Q® water were prepared for UV exposure experiments. The entire suspensions were poured into a glass Petri dish containing 0.053 M phosphate buffered water. The total volume in the Petri dish after adding oocysts suspensions was 20 mL. The oocysts suspensions were vortexed for 30 seconds before pouring into the Petri dish. Prior to the UV exposure, the suspensions in the Petri dish were mixed thoroughly at a faster rate using a 3 mm x 10 mm Teflon® coated stir bar. During the UV exposure, the suspensions were stirred slowly to allow gentle and continuous mixing. The UV irradiance at the surface of the liquid and at the centre of the Petri dish was measured for each exposure setting with a calibrated radiometer and detector (P-9710 Optometer and UV-3710 Irradiance Detector, Gigahartz Optik, Newburyport, MA). This measurement was corrected for radial variation of irradiation at the liquid surface, reflection at air water interface, absorbance and divergence within the water column. The correction factors were determined according to the methods described by Bolton and Linden (2003). For the phosphate buffer suspension, absorbance at 254 nm was measured by using a conventional UV-Vis spectrophotometer (Ultorspec 2000, Pharmacia Biotech, Cambridge, England). Since low pressure lamps were used for UV source, the entire output of the UV lamps was assumed to be at 254 nm and hence the sensor factor was assumed to be 1.0. The germicidal irradiance, E_{avg} (mW/cm^2) was calculated according to Equation 2-5 and the exposure time required to achieve a target UV dose was determined (Equation 2-6). The exposure time was controlled by a pneumatically driven automatic shutter equipped with a timer.

Following UV exposure, the entire suspension in the Petri dish was transferred to a 40 mL Teflon® tube. The Petri dish was washed once with 20 mL of milli-Q® water and the wash water was also transferred to the Teflon® tube. Four UV doses; 1, 5, 10 and 40 mJ/cm^2 were evaluated for the phosphate buffered suspension. Exposures were carried out in duplicate for 1 and 40 mJ/cm^2 and in triplicate for 5 and 10 mJ/cm^2.

3.3.3 Filtered water

The effect of aggregation on UV inactivation on C. *parvum* oocysts in filtered water was evaluated at two UV doses; 5 and 40 mJ/cm². For target dose of 5 mJ/cm², two independent filtration runs were conducted whereas for 40 mJ/cm², only one filtration run was possible because of oocyst stock limitation. A homogenizing step was introduced in order to disrupt the oocyst-particle or oocyst-oocyst aggregation before or after UV exposure. The procedure for homogenization will be described later. Seeded filtrate collected from the filtration experiment was split into six sub samples; each sub sample containing approximately 300 mL of filtered water. Prior to splitting, the entire seeded filtrate was stirred gently in order to ensure that each sub sample has identical oocysts concentration. Three of the six sub samples were homogenized before UV exposure (H+UV) and the other three were homogenized after UV exposure (UV+H). For the filtered water exposed to 5 mJ/cm², the UV exposure experiments were carried out in the same way as phosphate buffered water with some slight modifications. Any vortex or fast stirring which might disrupt the particle association of the oocysts in filtered water was avoided particularly for the UV+H samples. Absorbance of the sub samples at 254 nm was measured by a UV-Vis spectrophotometer (UV-2401 PC, Shimadzu Corp., Columbia, Maryland) equipped with integrating sphere attachment (ISR 2200). The integrating sphere attachment measures both transmitted and scattered light and hence it provides more accurate measurement of the absorbance of the suspension that contains light-scattering particles compared to a conventional transmittance spectrophotometer. Each sub sample of the seeded filtrate was exposed to UV irradiation in Petri dishes. Since a Petri dish can contain only 20 mL of suspension, at least 15 trials were required to expose the entire suspension of a sub sample to UV irradiation. The sub samples were not concentrated prior to UV exposure because the techniques available for concentration (i.e. centrifugation, membrane filtration) might disrupt or promote the oocyst aggregation. For the filtered water exposed to UV dose of 40 mJ/cm², the exposure experiments were carried out in 150 mL beakers. Beakers were used in order to reduce the number of exposures because the time required to deliver a germicidal dose of 40 mJ/cm² was very high (approximately 40 min). Beakers (150 ml, 5.2 cm internal dia) were filled with 150 mL of filtered water and then exposed to UV in the collimated beam apparatus. The suspension was stirred gently during exposure in order to ensure complete mixing. The time required to deliver the target dose was calculated based on the methods described by Bolton and Linden, (2003) . The freeboard above the water surface in the beaker was very small (~0.5 cm) and hence the impact of internal reflection of UV light from the sides of the beaker to the water was minimal. Following UV exposure, all 6 sub samples were filtered through 0.8 µm polycarbonate track etched (PCTE) membrane filters (Isopore™ membrane filters, Millipore, Ireland) by using a membrane filtration glassware and a vacuum filtration apparatus. The oocysts retained on the filter were washed off by scraping with a rubber policeman and suspended to 40 mL of milli-Q® water. The suspension was collected into a Teflon® tube and store in 4°C for infectivity assay. After filtration, the UV experiments were completed as quickly as possible (within 24 hrs) in order to avoid any settling of the filtered water.

3.3.4 Homogenization

A tissue homogenizer (PowerGen 700, Fisher Scientific, Pittsberg, PA) was used to disrupt the oocyst aggregation before or after UV exposure. The homogenizer had a 7 mm diameter shaft which was protected by a generator tube. The shaft, equipped with a rotor knife (6 mm dia and 12.7 mm long) at one end could rotate at 6 different speeds ranging from 10,000 to 30,000 rpm. During operation, the suspended particles were drawn into the core of the homogenizer through the narrow slits at the end of the generator tube and aggregates were broken down by the shearing action of the knife. All the samples were homogenized in a 500 mL conical flask at 30,000 rpm for 3 min before or after the UV exposure. The effect of aggregation on UV inactivation of *C. parvum* in filtered was evaluated by comparing log inactivation of the oocyst homogenized before and after UV exposure.

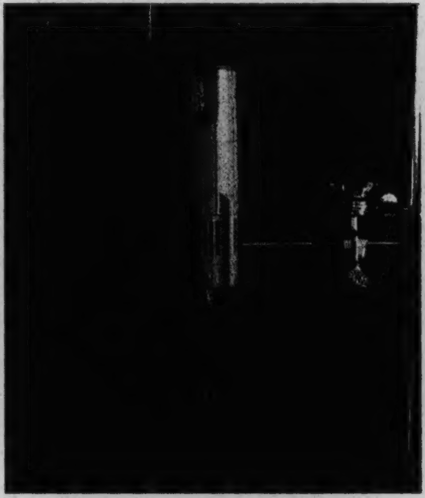


Figure 3.4 Tissue homogenizer

3.3.5 Controls

Two types of control experiments; positive and negative, were carried out along with the UV exposure experiments. For positive or zero dose controls, phosphate buffered water or filtered water suspensions were subjected to all the processing and handling steps as the experimental suspensions except that they were not exposed to UV irradiation. Negative controls were carried out by inoculating the chamber well slides with heat inactivated oocysts (approximately 10,000 oocysts per well). Heat inactivated oocysts were prepared by placing a 15 mL conical tube containing live oocyst suspension into a water bath at 70°C for 30 min.

3.4 *C. PARVUM* OOCYSTS PRODUCTION

C. parvum oocysts, used for developing dose-response relationship and UV exposure experiments were produced using previously established method (Craik et al., 2001). Holstein calves; aged 2 to 4 days were infected with 100×10^6 to 150×10^6 oocysts and were maintained on a diet of electrolyte solution. Feces collected from the calves were passed through a series of sieves (425μm - 75μm) and concentrated by centrifugation. Oocysts from the feces were separated by cesium chloride gradient centrifugation. Separated oocysts were washed by 0.01% Tween 20 and collected by centrifugation. Stock suspensions of purified oocysts were stored at 4°C in milli-Q® water with antibiotics (penicillin and streptomycin). During the period of the study, 5 batches of *C. parvum* oocyst were produced. However, only two batches provided enough viable oocysts for the UV exposure experiments.

3.5 IN VITRO CELL CULTURE

An in vitro cell culture method was used in this study for determining the inactivation of *C. parvum* oocysts following UV exposure. Cell culture methods have been reported to correlate well with the "gold standard" mouse infectivity method (Rochelle et al., 2002a; Slifko et al., 2002). Moreover, these methods are sensitive and produce results more rapidly and less expensively when compared to the mouse infectivity method. The cell culture method used in this study is adopted from Slifko et al. (1997) and referred as cell culture–foci detection method (CC-FDM). A brief description of the method is provided in subsequent sections.

3.5.1 Cell growth and maintenance

Human illeocecal adenocarcinoma cells (HCT-8 cells) (ATCC, Manassas, VA) were maintained in 75 cm^2 tissue culture flasks in RPMI 1640 medium (GibcoBRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA.), 1% 200 mM L-glutamine (Sigma, St. Louis, MO) and 2% 1 M HEPES (Fisher Biotech, Fair Lawn, NJ). Antibiotic (penicillin and streptomycin, Sigma, Oakville, ON) was added (2 mL/100 mL) in order to prevent any bacterial growth in the complete medium. The flasks were incubated in a 5% CO_2 atmosphere at 37°C and the HCT-8 cells were passaged every 2 or 3 days. The cells were passaged with one part phosphate buffered saline (PBS)-EDTA and one part 0.25% trypsin solution. HCT-8 cells were incubated for 10 to 15 minutes in trypsin–EDTA solution at 37°C in 5% CO_2 atmosphere to assist in the disruption of the monolayer. After incubation, the cell suspension was transferred to a 15 mL corning® tube and was pipetted vigorously with a 1 mL pipette tip in order to separate the cells. The cells were concentrated then by centrifugation (200 × g, 4 min, 4°C), resuspended in 5 mL of complete medium (the same medium used for cell growth in 75 cm^2 tissue culture flasks) and transferred into new flask (1mL for each flask) containing 30 mL of complete medium at each passage. For the FDM method, LabTech II (Nalgene Nunc, Naperville) 8 well chamber slides were seeded with 5×10^5 cells per well and the cell monolayer was grown to approximately 60 to 80% confluency in a 5% CO_2 atmosphere at 37°C for 24 to 48h. The working volume of each well was 400 μL.

3.5.2 Oocyst preparation

Oocyst suspensions from UV exposure and control experiments were collected in Teflon® tubes and centrifuged (27000×g, 10 min, 4°C). Supernatant was discarded and the oocysts were resuspended in milli-Q® water in microcentrifuge tubes. Following another centrifugation (10,500 ×g, 4 min, 4°C), the oocysts were resuspended in 1 mL of 10% (vol/vol) bleach (5.25% sodium hypochlorite) and were kept on ice for 8 to 10 min. For the logistic dose response experiments, oocysts from stock suspension were directly added to the bleach solution and kept on ice for 8 to 10 min. The purpose of this bleach pretreatment was to induce excystation and to kill bacteria and fungi which might contaminate the monolayer. The bleach solution was centrifuged (10,500 ×g, 4 min, 4°C) and the supernatant was discarded. The oocysts were then washed twice with sterile milli-Q® water and were resuspended in 1 mL of complete medium at room temperature. The oocysts suspension was then enumerated in duplicate using a hemacytometer. The hemacytometer counting was performed in accordance with the USEPA's method 1623 (USEPA, 2001). For oocysts that were recovered from filtered water, plain hemacytometer counting was prone to error because of the interference from the particles that were concentrated along with the oocysts. To assist in counting, oocysts in a sub sample of the filtered water suspension were stained with fluorescein-labeled mouse monoclonal antibody (A100 FLR, Aqua-Glo™, Waterborne, Inc, New Orleans, LA). Stained oocysts were then counted them using a hemacytometer under epi fluorescence microscopy. The general procedure was to prepare two dilutions (1:10 to 1:20) of the oocysts suspension (total volume 100 µL) in complete medium containing 45 µL of Aqua-Glo™ antibody reagent. The dilutions were vortexed for 30 sec and then incubated in a 5% CO_2 atmosphere at 37°C for 30 min. Following incubation, the oocysts in each dilution were counted in duplicate.

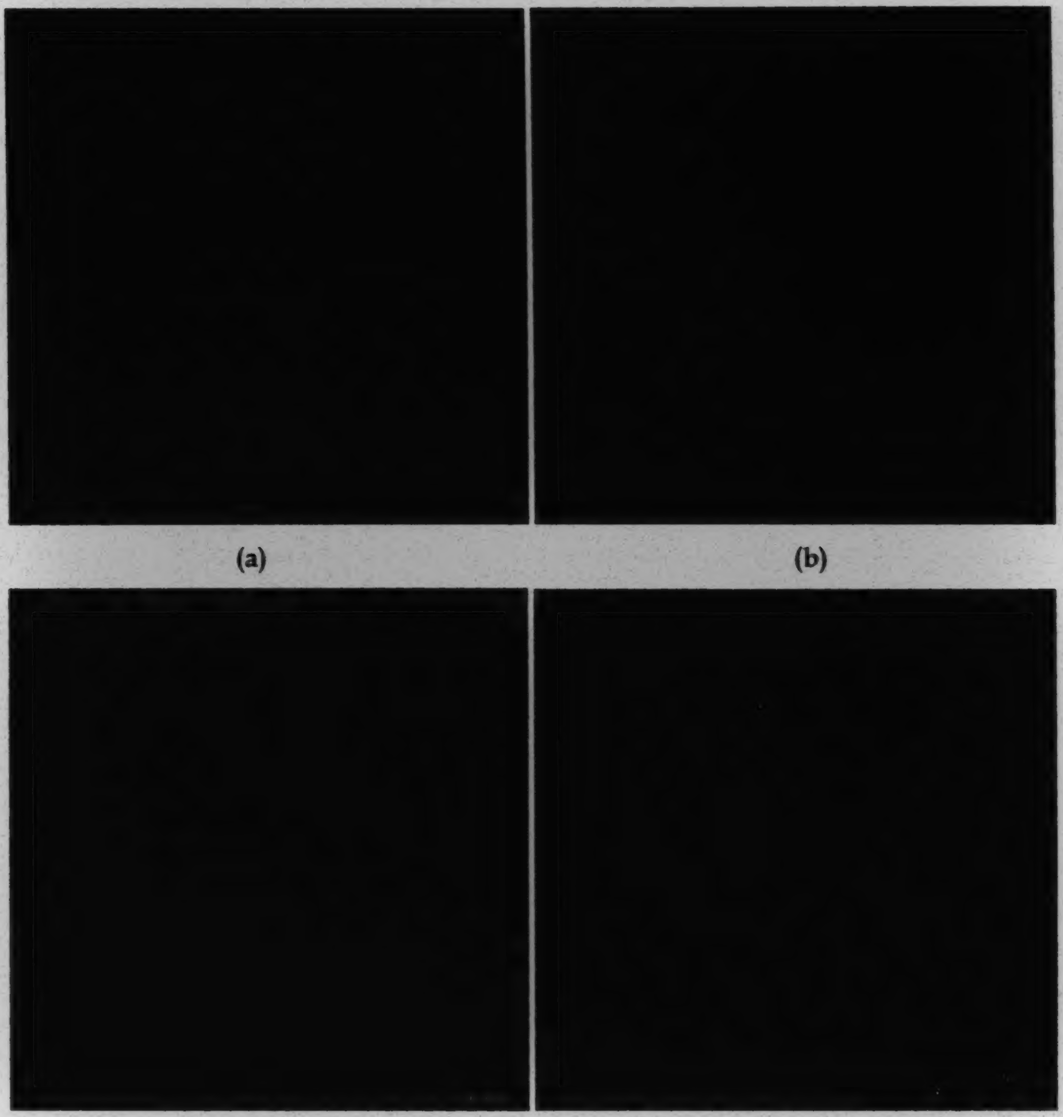
3.5.3 Cell infection

Following incubation, maintenance medium was aspirated from the wells and the cell monolayers were washed once with sterile 1×PBS. Dilutions of oocyst suspensions were prepared and the targeted number of ocysts was added to the monolayers. The chamber slides were incubated for 90 min in a 5% CO_2 atmosphere at 37°C in order to initiate excystation and infection. Oocyst debris and any unexcysted oocysts were removed from the monolayers by washing with 1×PBS. After washing, complete medium (400 µL) was added and the cells were incubated in 5% CO_2

atmosphere at 37°C for 48 to 72h. During the incubation period, the chamber slides were monitored routinely to assess the structural integrity of the monolayers.

3.5.4 Foci Detection Method

Following incubation, the maintenance medium was removed by aspiration and the cell monolayers were washed with 1×PBS and fixed with 100% methanol for 10 min. The chamber wells were removed and the slides were rehydrated at room temperature for 30 min with blocking buffer containing 1% normal rabbit serum (Sigma, St. Louis, MO) in 1×PBS. A fluorescein - labeled antibody reagent (A600-FL, Sporo-Glo™, Waterborne Inc., New Orleans, LA) was applied to the cell monolayers and the slides were incubated in a humid chamber for 60 min. The Sporo-Glo™ antibody reagent consists of a fluorescein-labeled rat anti-*C. parvum* sporozoite polyclonal antibody which binds with sporozoites, merozoites and all other intracellular reproductive stages. These life stages appear bright apple green when viewed under epi-fluorescence microscopy. Following incubation, the slides were rinsed by gently soaking them in a beaker containing 1×PBS for 3 min. A drop of BlockOut™ Counterstain (Waterborne, Inc., New Orleans, LA) was applied to each well and the slides were rinsed again with 1×PBS after 1 min incubation. The counterstain reduces non specific background fluorescence and creates a reddish background to enhance contrast during detection of infectious foci. The slides were then air dried and coverslips were placed following application of No-Fade™ Mounting Medium (Waterborne, Inc., New Orleans, LA). The slides were then mounted in an epi-fluorescence microscope and thoroughly examined at 200× magnification. Infection on the monolayers was detected when a bright apple green cluster of *C. parvum* life stages were observed (figure 3.2). If clustering was observed, it was confirmed at 400 × magnification and the well was marked as positive. The wells were marked as negative if no or pin point invasion was observed. Pin point invasion was created by UV inactivated oocysts which might have invaded the cell lines but could not go through the life stages. Figure 3.4 shows the images of different types of *C. parvum* infection on the cell monolayers as observed under epi-fluoresce microscopy.



(a) (b)

(c) (d)

Figure 3.5 Confocal microscopic images of infected and non-infected cell monolayers showing (a) clustering following inoculation with UV exposed oocysts, (b) pinpoint invasion following inoculation with UV exposed oocyst, (c) clustering following inoculation with 25 live oocysts and (d) negative control. All the slides were incubated at 37°C and 5% CO_2 atmosphere for 48-72 hours.

3.6 INFECTIVITY ANALYSIS USING LOGISTIC DOSE-RESPONSE MODEL

Inactivation of the C, *parvum* oocysts following UV exposure was determined based on reduction in infectivity of the oocysts. The log inactivation was calculated as,

$$-\log\left(\frac{d}{d_0}\right) \tag{3-1}$$

where, *d* was the number of infectious oocysts in the inoculum and d_0 was the total number of oocysts in the same inoculum. The number of infectious oocysts (*d*), in the inoculum was estimated based on a logit dose-response relationship,

$$\ln\left[\frac{P}{(1-P)}\right] = \beta_0 + \beta_1 \log d \tag{3-2}$$

Where, *P* was the proportion of wells in which positive infections were detected following inoculation with *d* oocysts and β_0 , β_1 are the parameters of the logit model. Separate dose response relationships were developed for the two batches of oocysts used for the UV exposure experiments. Oocyst doses ranging from 10 to 200 oocysts were added to 8 well chamber slides and the number of wells infected for each dose was determined by the FDM method as described earlier. The dose-response trials were carried out along with UV exposure trials and the data were pooled together to develop one logistic model for each batch of oocysts. The parameters of the logit model were estimated by maximizing the ln likelihood function:

$$\ln L = \sum_{i=1}^{\lambda} Y_i(\beta_0 + \beta_1 X_i) - \sum_{i=1}^{\lambda} \ln\left[1 + \exp(\beta_0 + \beta_1 X_i)\right] \tag{3-3}$$

where, the subscript i =1, 2, . . ., λ denotes each individual well used in the dose response experiment, X_i is the log of the oocyst dose for each well, and Y_i is the binary score (0 for negative infection, 1 for positive infection) of each well. The likelihood function was maximized using the Solver tool in Microsoft Excel™. Individual and confidence interval of the parameters at (1-α) significance level were constructed using the likelihood ratio test (Seber and Wild, 1989):

$$\ln L(\beta) - \ln L_{\max}(\beta_{1-\alpha}) \leq \frac{\chi^2_{p,\alpha}}{2} \tag{3-4}$$

Where, p = number of parameters, β is the parameters of the logit model and $\chi^2_{p,\alpha}$ is the Chi-square distribution.

3.7 IMPACT OF HOMOGENIZATION ON THE INFECTIVITY OF *C. PARVUM* OOCYST

In order to evaluate the impact of the homogenization technique on the infectivity of C. *Parvum,* live oocysts were suspended in 150 ml filtered water and homogenized at 30,000 rpm for 3 minutes. Following homogenization, the oocyst suspension was concentrated using membrane filtration and was stored at 4°c for cell culture infectivity assay. The cell culture infectivity assay was performed as described in section 3.5 and the proportion of wells infected for a particular oocyst inoculum was determined. A similar infectivity assay was carried out using oocysts suspended in either milli-Q® or filtered water but that were not homogenized. The counting method used for oocysts supended in milli-Q® water was direct hemacytometer counting whereas the counting method used for oocysts suspended in filtered water was the ifa based hemacytometer counting described in section 3.5.2.

3.8 DIRECT MEASUREMENT OF OOCYST AGGREGATION

The number of oocysts associated with different particle size fractions was determined by serial filtration of the filtered water. Approximately 1L of filtered water collected from filtration runs no. 1, 2 and 6 was serially filtered through 10, 5, 2, and 0.8 µm pore size polycarbonate track etched (PCTE) membrane filters (Isopore™ membrane filters, Millipore, Ireland) by using a membrane filtration glassware and a vacuum filtration apparatus. The particles retained on each filter were washed off by gentle scraping with a rubber policeman and were suspended in 40 mL of milli-Q® water. After homogenizing, the water was collected in a Teflon® tube and the tube was centrifuged (27000×g, 10 min, 4°C). Supernatant was discarded and the oocysts from each tube were resuspended in 1 mL of milli-Q® water in a microcentrifuge tube. The oocysts in each microcentrifuge tube were concentrated again by centrifugation (10,500 × g, 4 min, 4°C) and the supernatant was discarded. 45 µL of Aqua-Glo™ antibody reagent was applied to the oocysts and the suspension was incubated in 5% CO_2 atmosphere at 37°C for 30 min. Following incubation, the oocysts in the suspension were counted in duplicate using a hemacytometer under epi-fluorescence microscopy. The total number of oocysts in the suspension was determined by measuring the volume of the suspension using a 200 µL pipette. If the oocysts concentration was too high, dilutions were prepared and counted. Theoretically, oocysts captured at different cut-off filters were associated with particles larger or equal to those cut-off sizes. For control, oocysts suspended in milli-Q® water were homogenized (3000 rpm, 3 min) and were filtered serially through the PCTE membrane filters. The oocysts captured at different cut-off sizes were counted.

3.9 MICROSCOPIC EXAMINATION OF OOCYST AGGREGATION

3.9.1 Scanning Electron microscopy (SEM)

Approximately 1 L of filtered and backwash water were filtered through PCTE membrane filters of different size (10, 5, 2 and 0.8 µm) and the filters were placed in 150 × 15 mm polystyrene Petri dishes. The filters were fixed overnight with 2% glutaraldehyde. Square sections (15 × 15 mm) were cut from the filters and washed with 1×PBS twice (30 min each). Then the filter sections were rinsed in a series of 20, 30, 50, 70, 90 and 100% ethanol solutions for 15 min each. In between each rinse the samples were allowed to dry for 15 min. The sections were further rinsed with another series of 2:1, 1:1 combination of 100% ethanol and hexamethyldisilazane (HMDS) for 15 min each. Finally, the sections were washed twice with HMDS and air dried. The dried filter sections were then attached to a metal stud and sputtered with thin layer of gold. The gold coated filters were mounted on a SEM (Philips / FEI LaB6 Environmental Scanning Electron Microscope) and examined. SEM was carried out at the Advanced Microscopy Facility in the Department of Biological Sciences at University of Alberta.

3.9.2 Confocal microscopy

Filtered water (150 mL) was passed through 0.8 μm PCTE membrane filters and the filters were collected in 150 × 15 mm polystyrene Petri dishes. Milli-Q® water (1 mL) was added to the filter surface and the Petri dishes were rocked gently for 15 min in order to dislodge the particles from the filter surfaces. The suspension containing particles and oocysts was then transferred to microcentrifuge tubes. Approximately 50 μL of the suspension from each tube was immediately added to a 9 mm Dynal® well slide (Dynal Biotech ASA, Oslo, Norway). Vortexing or any other shaking of the tubes was avoided in order keep the oocyst aggregation intact. The slides were then air dried in a slide warmer. Aqua-Glo™ antibody was added to each slide and spread uniformly over the entire well. The slides were then incubated in 5% CO_2 atmosphere and at 37°C for 30 min. Following incubation, the antibody was washed off from the slides by rinsing with 1×PBS. Mounting media was applied and the slides were examined using confocal microscopy (Leica TCS-SP2 Multiphoton Confocal Laser Scanning Microscope). In confocal microscopy, fluorescence images (excitation at 480 nm, emission at 520 nm) and differential interference contrast (DIC) imaging of the wells was done simultaneously using two different microscope channels. The superimposed images were examined for detecting oocysts aggregation in filtered water. Confocal microscopy was carried out at the Advanced Microscopy Facility in the Department of Biological Sciences at University of Alberta.

4.0 ANALYSIS OF THE RESULTS

This chapter presents the experimental results of the study and their analysis. This chapter is divided into four sections: logistic dose-response models, impact of homogenization on the infectivity of *Cryptosporidium parvum* oocysts, water treatment process simulation, UV exposure experiments, direct measurement of oocysts aggregation, and microscopic examination of oocyst aggregation.

4.1 LOGISTIC DOSE RESPONSE MODEL

Two batches of C. *parvum* oocysts were used for the UV exposure experiments in this study. The oocyst batches were produced from Holstein claves and stored at 4°C in milli-Q® water with antibiotics. Cell-culture logistic dose-response model (Equation 3-2) for both batches of oocyst was developed and used for determining the number of infectious oocyst present in the inoculums following UV exposure. The dose response models for the two batches of oocysts along with their 90% confidence limits are shown in Figures 4.1 and 4.2. Experimental data including oocyst age and the proportions of wells infected at a particular oocyst dose for all the dose response trials is provided in Tables A-1 and A-2 (Appendix A). There was no evidence of a correlation between oocyst infectivity and oocyst age. Nevertheless, most of the dose-response trials were carried out at the same time as the UV exposure trials and were spread over the life of the batches (0-4 months for batch no. 1 and 1-4 months for batch no, 2). Consequently, the effect of oocyst age (if any) was averaged out in the dose-response models developed for the two batch of oocysts. The value of the model parameters and their individual 90% confidence interval are summarized in Table 4-1. The ID_{50} (the dose at which 50% of the wells became infected) of the two oocyst batches is also shown in Table 4-1. Values of the parameters, β_0 and β_1 were estimated using maximum likelihood method (Equation 3-3) and their 90% confidence limits were estimated by the likelihood ratio test (Equation 3-4). The 90% confidence interval of the model parameters β_0 and β_1 of the two dose response model overlaps with each other (Table 4-1). This suggests that the difference between the dose response models of the two oocyst batches was not statistically significant at the 90% confidence level.

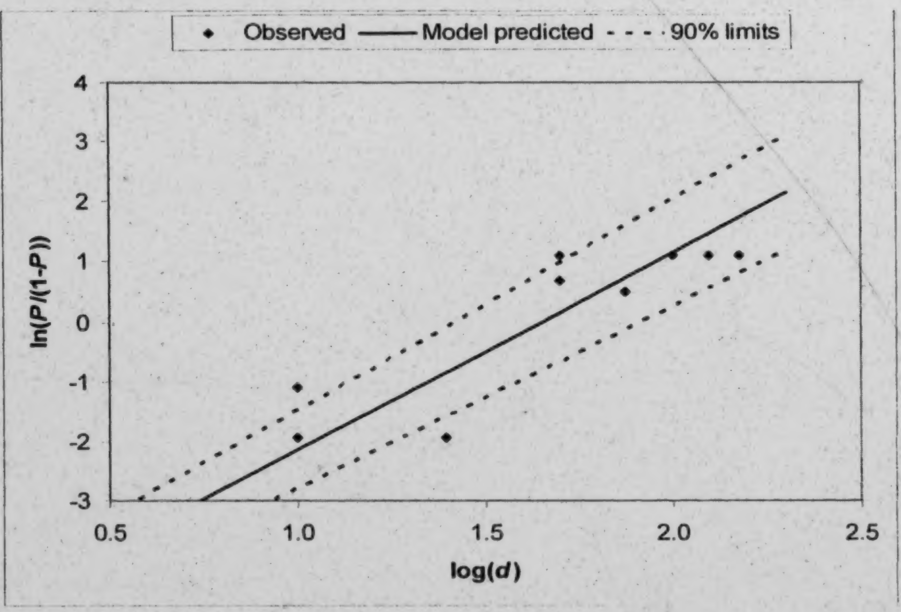


Figure 4.1 Cell culture logistic dose-response model for oocysts from batch #1

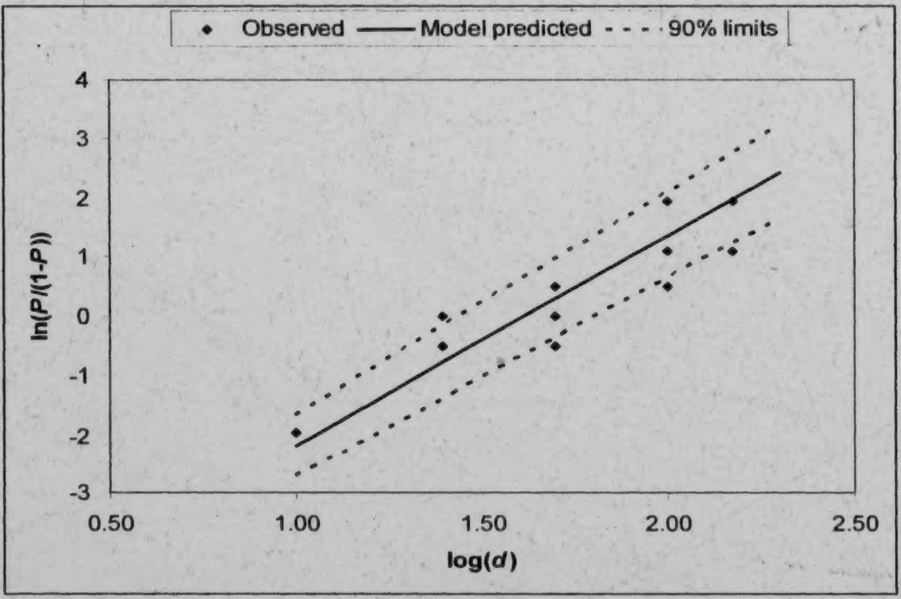


Figure 4.2 Cell culture logistic dose-response model for oocysts from batch #2

Table 4-1 **Logistic dose response models of *C. parvum* oocysts in cell culture**

Oocyst batch no.	β_0	90% limits of β_0	β_1	90% limits of β_1	ID_{50}
1	-5.43	-5.8, -5.0	3.29	3.1, 3.5	45
2	-5.71	-6.0, -5.4	3.54	3.4, 3.7	41

The ID_{50} of batch No. 1 and 2 were calculated as 45 and 41 oocysts respectively. This is comparable to the ID_{50} reported by other researchers using a similar in vitro cell culture infectivity assay. Slifko et al. (2002) reported that the ID_{50} of C. *parvum* oocysts in HCT-8 cell line ranged from 5 to 50 oocysts. The foci detection method (FDM) was used for detection of infectious oocyst in that study. In another study, however, using cell culture based RT-PCR method, Rochelle et al. (2002) estimated the ID_{50} of C. *parvum* oocyst in HCT-8 cell line as 81 to 106 oocysts. The difference between the two sets of results may be related to the method used to detect infections; however, oocyst infectivity may be influenced by a number of other variables including oocyst age, host and statistical method used for parameter estimation.

4.2 IMPACT OF HOMOGENIZATION ON THE INFECTIVITY OF *C. PARVUM* OOCYST

Homogenization was used to break up aggregates and disperse C. *parvum* oocysts before or after UV exposure. However, it was possible that the rotor knife of the homogenizer that breaks up the aggregates during homogenization might physically damage the oocyst wall and hence it might affect the infectivity of the oocysts. In addition, the rise in temperature of the suspension due to homogenization might also affect the infectivity of the oocysts. Results from the cell culture infectivity assay with oocysts that were suspended in homogenized or non homogenized filtered water and milli-Q water are summarized in Table 4-2. Analysis of variance (ANOVA) suggested that the effect of homogenization on the infectivity of the oocysts, as measured by the percent of wells infected in cell culture was not significant at the 95% confidence level ($p = 0.29$). ANOVA also indicated that the difference in hemacytometer counting procedure between milli-Q and filtered water did not affect the infectivity assay significantly at the

95% confidence level (p = 1). The age of the oocyst stock when this experiment was conducted was 7 months and therefore the infectivity of the oocysts stock was lower than anticipated. However, since this experiment was only used for the purpose of comparison, the loss of infectivity of the oocyst over time is unlikely to affect the outcome.

Table 4-2 Effect of homogenization on the infectivity of *C. parvum* oocysts

Trial no	Type of suspension	Oocyst dose	Proportions of well infected	Percent wells infected
1	Milli-Q water	200	5/8	62.5
4	Milli-Q water	200	7/8	87.5
7	Milli-Q water	200	8/8	100
2	Filtered water (homogenized)	200	6/8	75
5	Filtered water (homogenized)	200	8/8	100
8	Filtered water (homogenized)	200	6/8	75
3	Filtered water (non homogenized)	200	7/8	87.5
6	Filtered water (non homogenized)	200	4/8	50
9	Filtered water (non homogenized)	200	5/8	62.5

4.3 WATER TREATMENT PROCESS SIMULATION EXPERIMENTS

Six filtration runs were carried out during the period of the study. As mentioned earlier, a "filtration run" refers to a complete simulated water treatment process run which includes coagulation, flocculation, settling and sand-anthracite media filtration. The type of oocysts and raw water samples used for each filtration run and the experiments conducted with the filtered water collected from each filtration run are provided in Table 3.1. Seeded/settled water was added to the filter influent during the middle of a filtration run. After allowing sufficient time for the seeded water to pass through the filter media, a sample of seeded/filtered water was collected. The optimal collection time of the seeded /filtered water was previously determined using a tracer test conducted on the filter column using methylene blue. The details of the tracer test are provided in Appendix B.

Table 4-3 shows the turbidity, pH and temperature measured at different points of a typical filtration run (filtration run no. 4). Figure 4.3 and 4.4 shows the turbidity and particle count (>2 μm) as a function of time for the same filtration run. pH and temperature of raw water and unseeded settled water were recorded only for filtration runs where live oocysts were used for seeding. The turbidity and particle count data of all the filtration runs are provided in Appendix B. From Table 4.3, it can be observed that despite having a large oocyst concentration in the influent, the turbidity of the seeded/ filtered water was very close to the turbidity of unseeded effluent. The addition of alum and polymer in the raw water during the coagulation step decreased the pH slightly in the settled water. There was also a slight increase in water temperature which might have resulted from the rapid and slow mixing during coagulation and flocculation. However, it has been demonstrated that coagulation with alum at the concentration, pH and temperature utilized in this study does not have any significant effect on the viability of the oocysts (Robertson et al., 1992).

Table 4-3 Turbidity, pH and temperature of water measured at different point during filtration run #4

Point of measurement	Turbidity[1]	pH	Temperature (°C)
Raw water	6.21	7.8	9.1
Raw water after seeding with oocysts (500 × 10^6)	8.62		
Settled water (seeded)	8.05		
Settled water (unseeded)	5.31	6.5	14.2
Filtered water (seeded)	1.05		
Filtered water (unseeded)	0.96		

[1] Average of minimum 5 measurements

Figure 4.3 and 4.4 show the variation of turbidity and particle count (≥2 μm) with time respectively for filtration run no. 4. The time and duration of filtering seeded settled water in between filtering unseeded settled water is shown in Figure 4.3. Although the seeded settled water was initially spiked with 500 × 10^6 oocysts, filtered water turbidity did not increase significantly during the filtration of seeded settled water. The time dependent turbidity measurements of the filter effluent could not identify the point of oocyst breakthrough during the filtration run. In contrast, particle count (Figure 4.4) of the filter effluent started to increase a few minutes after the start of seeded water filtration and continued to increase along with the filtration run. The particle count measurements appeared to indicate the oocyst breakthrough more accurately than turbidity.

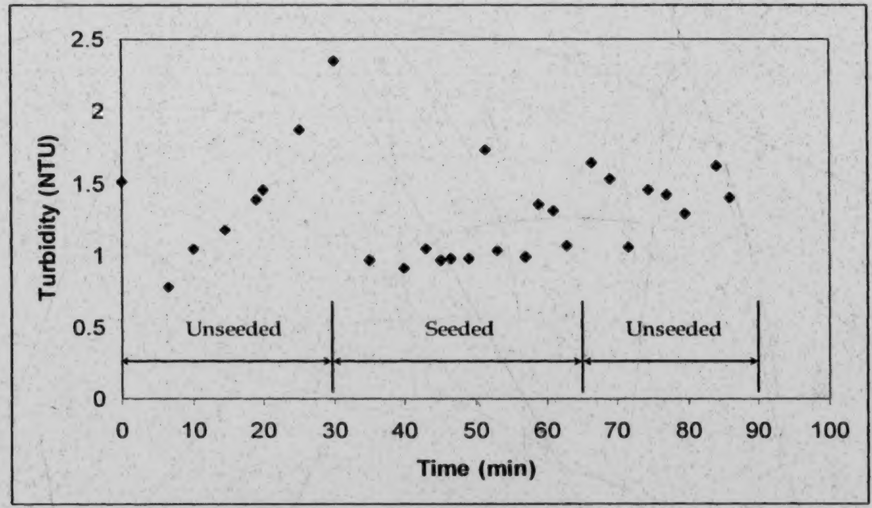


Figure 4.3 **Turbidity of the filtered water as function of time for filtration run no.4. Time zero corresponds to the start of the filtration run.**

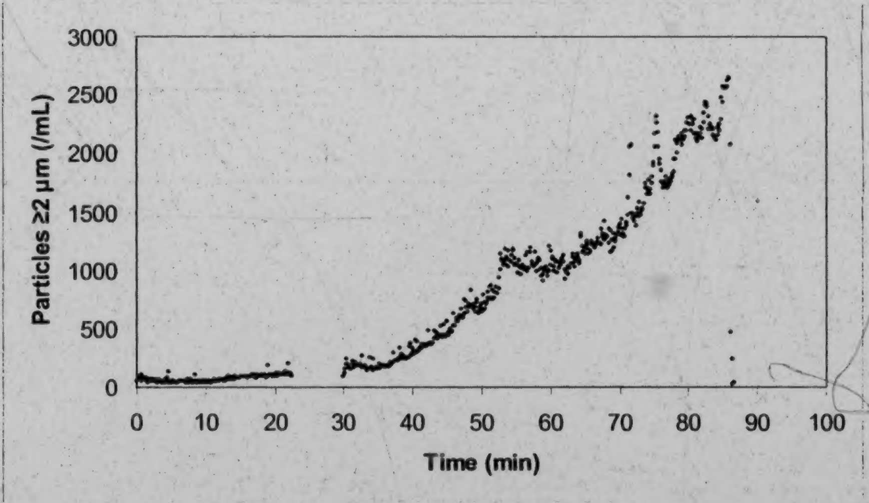


Figure 4.4 **Particle concentration of the filtered water as function of time for filtration run no. 4. Time zero corresponds to the start of the filtration run.**

4.4 UV EXPOSURE EXPERIMENTS

UV exposure experiments were carried out with C. *parvum* oocysts either suspended in phosphate buffered water or captured in filtered water. Results from the all the UV exposure trials including germicidal UV irradiance, exposure times and UV dose are presented in Tables 4-4 to 4-6. For UV exposure trials involving oocysts suspended in phosphate buffered water, oocyst batch 1 was used and for UV exposure trials involving oocysts collected in filtered water, oocyst batch 2 was used. Detailed information regarding the UV exposure conditions is provided in Appendix C.

The absorption coefficient of the test suspension varied with the type of water and the number of oocysts seeded in the raw water prior to coagulation. The absorption coefficient of phosphate buffered water suspension ranged from 0.041 to 0.06 cm^{-1}. The absorption coefficient of filtered water collected from filtration run no 3 and 4 ranged from 0.0665 to 0.106 cm^{-1} and 0.0805 to 0.96 cm^{-1} respectively. Since the raw water for both filtration run no 3 and 4 were seeded with same number oocysts (5×10^8), these absorption coefficient values were comparable. For filtration run no 5, the raw water was seeded with much higher (3×10^9) number of oocysts, resulting in a higher absorption coefficient of the filtered water (ranging from 0.159 to 0.171). However, the absorption coefficient values for phosphate buffered water were measured using conventional transmittance spectrophotometry whereas the absorption coefficient values for filtered water were measured using integrating sphere spectrophotometry. A comparison of the absorbance measured using the two spectrophotometery methods was made for filtered water collected from filtration run no 3 (data not shown). The absorption coefficient measured by the conventional transmittance spectrophotometry was approximately 30% higher than that measured by the integrating sphere spectrophotometery. It was, therefore, important to account for the scattering effect of particles on the incident UV radiation in order to determine the UV dose accurately. Integrating sphere spectrophotometry was used in all subsequent UV exposures of filtered water.

Infectivity results for each trial are reported in Tables 4-4 to 4-6 as the number of wells positive for infection in each of the three 8-well chamber slides that were inoculated with different numbers of oocysts (d_0) for each infectivity assay. Inactivation (-log d/d_0) was calculated for those inoculum levels (d_0) for which the infectivity result was in the measurable range i.e. number of wells positive for infection was greater than zero but less than the total number of wells inoculated. For trials in which the results from more than one slide were in the measurable range, an arithmetic average of the log inactivation ratio determined from each slide was reported. If none of the wells were found positive for infection in any three 8 well slides (0/8) used for each trial, the log inactivation ratio was reported as greater than (>) value that would be calculated if one of the wells had been infected in the slide with highest inoculum (1/8). Conversely, if all the wells were positive in each of the three slides, the result was reported as less than (<) the value if all but one of the wells were infected in the lowest inoulum slide were infected. In a trial where all the wells in the highest inoculum were infected (8/8) and no wells were infected in the lower inoculum (0/8), the log inactivation ratio was reported as the arithmetic mean of the inactivation ratios determined in the two previous cases.

4.4.1 Phosphate buffered water

Results from UV exposure trials with oocysts suspended in phosphate buffered water are provided in Table 4-4. From Table 4-4, it is evident that a high level of log inactivation could be achieved even at low UV doses. At 5 and 10 mJ/cm^2, the average log inactivation from triplicate analyses was 3.0 and 3.2 respectively. These values are in close agreement with past studies which observed the UV inactivation of *C. parvum* oocysts suspended in laboratory water or filtered water (Bukhari et al., 1999; Clancy et al., 2000; Craik et al., 2001; Mofidi et al., 2001; Shin et al., 2001; Zimmer et al., 2003; Rochelle et al., 2004). At high UV dose of 40 mJ/cm^2, none of the wells were infected for a inoculum size of 5×10^5 and the average log inactivation was estimated as >4.7. Figure 4.5 shows low-pressure UV inactivation of *C. parvum* observed in different studies as assessed by both in vitro cell culture and animal infectivity assays. From Figure 4.5, it can be observed that the log inactivation values of the oocysts obtained in this study correlate well with past studies. This suggests that the CC-FDM method used in this study was able to predict accurately the inactivation of *C. parvum* oocysts following UV exposure.

Table 4-4 **Results from UV exposure trials for oocysts suspended in phosphate buffered water**

Trial No.	Irradiance E_g (mW/cm^2)	Exposure Time, t (sec)	UV dose $E_g \times t$ (mJ/cm^2)	d_0	P	d	-log (d/d_0)	Avg -log(d/d_0)
1	0.071	14	1.0	5,000	8/8	>176	<1.5	0.8
				500	5/8	64	0.9	
				50	1/8	11	0.7	
6	0.037	27	1.0	5,000	8/8	>176	<1.5	0.7
				500	6/8	97	0.7	
				50	0/8	<11	>0.6	
2	0.0714	70	5.0	50,00	4/8	45	3.1	2.9
				5,000	1/8	11	2.7	
				500	0/8	<11	>1.7	
3	0.0715	70	5.0	50,00	3/8	31	3.2	3.2
				5,000	0/8	<11	>2.6	
				500	0/8	<11	>1.7	
7	0.037	136	5.0	50,00	3/8	31	3.2	2.9
				5,000	1/8	11	2.7	
				500	0/8	<11	>1.7	
4	0.0715	140	10.0	500,0	7/8	176	3.5	3.4
				50,00	2/8	21	3.4	
				5,000	0/8	<11	>2.7	
8	0.037	270	10.0	500,0	8/8	>176	<3.5	2.4
				50,00	7/8	176	2.5	
				5,000	2/8	21	2.4	
9	0.038	265	10.0	500,0	6/8	97	3.7	3.6
				50,00	2/8	21	3.4	
				5,000	0/8	<11	>2.6	
5	0.0549	728	40.0	500,0	0/8	<11	>4.7	>4.7
				50,00	0/8	<11	>3.7	
				5,000	0/8	<11	>2.7	
10	0.037	1077	40.0	500,0	0/8	<11	>4.7	>4.7
				50,00	0/8	<11	>3.7	
				5,000	0/8	<11	>2.7	

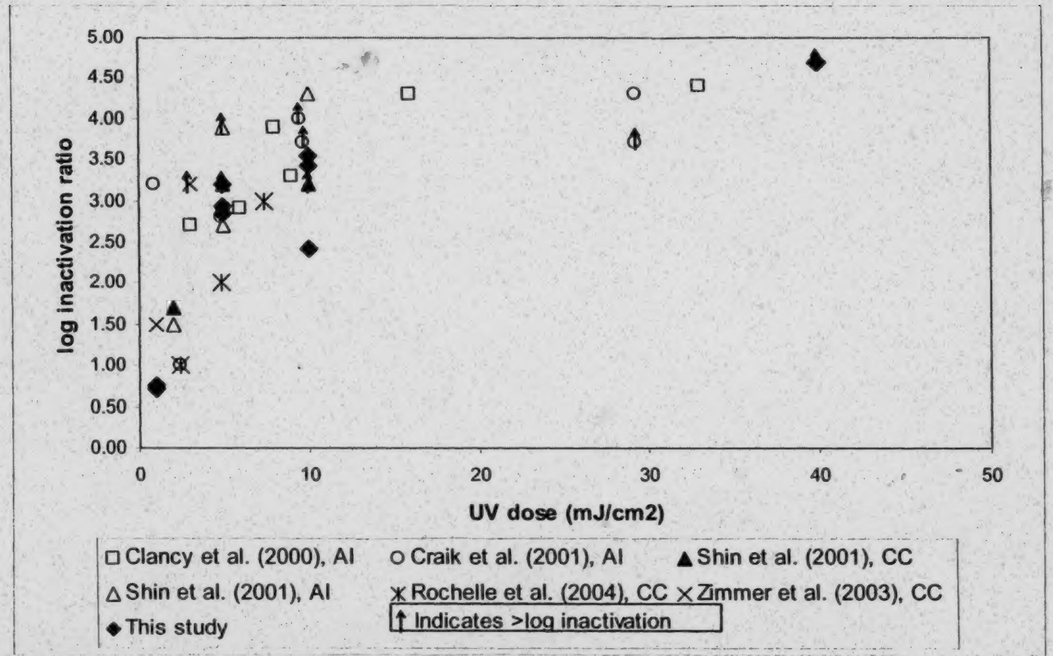


Figure 4.5 UV inactivation of *C. parvum* oocysts observed in different studies using low pressure lamps. AI indicates animal infectivity and CC indicates cell culture. The arrows indicate that actual inactivation was greater than the indicated data point (i.e. no infections were observed in either the cell culture wells or in the animals).

4.4.2 Filtered water

The effect of upstream treatment processes on UV inactivation of *C. parvum* was evaluated by exposing oocysts collected in filtered water to two UV doses; 5 and 40 mJ/cm^2. The UV dose of 5 mJ/ cm^2 was selected because at this dose the predicted log inactivation of the oocyts was expected to be within a measurable range (Table 4-4 and Figure 4-5) (i.e. 2 to 4 log). Also, in this range the level of inactivation is proportional to UV dose. A UV dose of 40 mJ/cm^2 was selected because almost complete inactivation of *C. parvum* has been observed at this dose level (Table 4-4 and Figure 4-5). In addition, many water treatment plants operate their UV disinfection systems around this dose level. Because of the relatively high level of inactivation expected, the oocyst seeding requirement for 40 mJ/cm^2 was very high (i.e. 3×10^9 oocysts) and was difficult to achieve. Therefore, only one filtration run was completed for this dose level.

Table 4-5 **Results from UV exposure trials for oocysts collected in filtered water from filtration run no. 3**

Trial No.	Sample type	Irradiance E_g (mW/cm^2)	Exposure Time, t (sec)	UV dose $E_g \times t$ (mJ/cm^2)	d_0	P	d	-log (d/d_0)	Avg. -log (d/d_0)
1	UV+H[1]	0.042	120	5.0	50,000	8/8	>146	<2.5	1.7
					5,000	5/8	57	1.9	
					500	2/8	20	1.4	
2	UV+H[1]	0.042	120	5.0	50,000	8/8	>146	<2.5	1.1
					5,000	8/8	>146	<1.5	
					500	4/8	41	1.1	
3	UV+H[1]	0.042	120	5.0	50,000	7/8	146	2.5	2.5
					5,000	2/8	20	2.4	
					500	0/8	<12	>1.6	
							Average		**1.7±0.22[3]**
4	H+UV[2]	0.042	119	5.0	50,000	7/8	146	2.5	2.2
					5,000	2/8	20	2.4	
					500	1/8	12	1.6	
5	H+UV[2]	0.04	125	5.0	50,000	4/8	41	3.1	2.7
					5,000	2/8	20	2.4	
					500	0/8	<12	>1.6	
6	H+UV[2]	0.042	120	5.0	50,000	5/8	57	2.9	2.7
					5,000	2/8	20	2.4	
					500	0/8	<12	>1.6	
							Average		**2.5±0.22[3]**

[1] Filtered water sample was homogenized after UV exposure

[2] Filtered water sample was homogenized before UV exposure

[3] Mean square error generated by ANOVA

Results from UV exposure trials for oocysts collected in filtered water from filtration run no. 3 and 4 are presented in Table 4-5 and 4-6 respectively. Approximately 2L of filtered water from each filtration run was split into six samples (three for homogenized before UV and three for homogenized after UV) and exposed to UV dose of 5 mJ/cm^2 in random order. The exposure times required to deliver UV dose of 5 mJ/cm^2 were calculated according to previously described method (section 3.3) for each sample. The average log inactivation values of filtered water samples (from triplicate analyses) that were homogenized after UV exposure (UV+H) for filtration runs no 3 and 4 were 1.7 and 1.5 respectively. For samples that were homogenized before exposure (H+UV), the average log inactivation values (from triplicate analysis) were 2.5 and 2.3 for filtration runs no 3 and 4 respectively. The reduction in log inactivation of oocysts in UV+H samples compared to H+UV samples was 0.8 for both filtration runs. When compared with phosphate buffered water, the reduction in log inactivation of oocysts in UV+H samples was 1.3 and 1.5 for the two filtration runs.

Table 4-6 **Results from UV exposure trials for oocysts collected in filtered water from filtration run no. 4.**

Trial No.	Sample type	Irradiance, E_g (mW/cm^2)	Exposure Time, t (sec)	UV dose $E_g \times t$ (mJ/cm^2)	d_0	P	d	-log (d/d_0)	Avg -log (d/d_0)
1	UV+H[1]	0.041	122	5.0	50,00	8/8	>146	<2.5	1.6
					5,000	8/8	>146	<1.5	
					500	1/8	12	1.6	
2	UV+H[1]	0.04	126	5.0	50,00	8/8	>146	<2.5	1.2
					5,000	8/8	>146	<1.5	
					500	3/8	29	1.2	
3	UV+H[1]	0.041	122	5.0	50,00	8/8	>146	<2.5	1.8
					5,000	3/8	29	2.2	
					500	2/8	20	1.4	
							Average		1.5±0.22[3]
4	H+UV[2]	0.04	126	5.0	50,00	7/8	146	2.5	2.2
					5,000	5/8	0	4.6	
					500	0/8	<12	>1.6	
5	H+UV[2]	0.039	127	5.0	50,00	5/8	57	2.9	2.8
					5,000	1/8	12	2.6	
					500	0/8	<12	>1.6	
6	H+UV[2]	0.04	124	5.0	50,00	8/8	>146	<2.5	1.9
					5,000	4/8	41	2.1	
					500	1/8	12	1.6	
							Average		2.3±0.22[3]

[1] Filtered water homogenized after UV exposure
[2] Filtered water homogenized before UV exposure
[3] Mean square error generated by ANOVA

A two factor analysis of variance (ANOVA) was performed on the log inactivation data of the two filtrations runs in order evaluate whether there was a statistically significant difference between log inactivation values of the filtered water samples homogenized before and after UV exposure and between the filtration runs . Two factor ANOVA considers the variability in the data set at once and allows investigation of interaction effects arising from different treatment conditions. Hence, the use of two factor ANOVA for the experimental data set is more appropriate than multiple one way ANOVA or isolated t-tests in individual treatment pairs. The basic assumptions ANOVA are that the values are independent and normally distributed and the variances are equal in each group (Montgomery, 2001). The results from ANOVA are summarized in Table B-1 (Appendix B). ANOVA revealed that the average log inactivation of UV+H and H+UV samples were significantly different at the 95% confidence level ($p = 0.022$). This indicates that there was a significant reduction in log inactivation of the *C. parvum* oocysts that were in a aggregated state in filtered water compared to the oocysts that were mono dispersed. There was no significant effect of filtration condition on UV inactivation of the oocysts. In order check the assumption of ANOVA, residual analysis was performed. Normal probability plot of the residuals (Figure B-1, Appendix B) confirmed that the data were normally distributed. Also, no indication of any unusual structure or trend of the residuals with respect to fitted values, filtration runs or sample types was observed (Figure B-2 to B-4, Appendix B). No statistical comparison was performed between the log inactivation values in phosphate buffered suspension and filtered water because of variation in experimental conditions i.e. oocysts batch, absorption measurement etc. However, the mean log inactivation of oocysts suspended in phosphate buffered water at 5 mJ/cm^2 was greater than that of filtered water samples homogenized before UV exposure. This indicates that the homogenization step may not have resulted in complete disaggregation and dispersion of the oocysts in the filtered water samples. Nevertheless, these findings are consistent with the original research_hypothesis that the aggregation of oocysts in filtered water affects the UV inactivation of these parasites.

Table 4-7 **Results from UV exposure trials for oocysts collected in filtered water from filtration run no. 5**

Trial No.	Sample type	Irradiance E_g (mW/cm^2)	Exposure Time, t (sec)	UV dose E_g x t (mJ/cm^2)	d_0	P	d	-log (d/d_0)	Avg -log (d/d_0)
1	UV+H[1]	0.016	2561.4	40.0	500,000	8/8	>146	<3.5	2.7
					50,000	5/8	57	2.9	
					5,000	2/8	20	2.4	
2	UV+H[1]	0.017	2408.5	40.0	500,000	6/8	84	3.8	3.4
					50,000	4/8	41	3.1	
					5,000	0/8	<12	>2.6	
3	UV+H[1]	0.015	2606.2	40.0	500,000	7/8	146	3.5	3.0
					50,000	4/8	41	3.1	
					5,000	3/8	29	2.2	
							Average		**3.0±0.28[3]**
4	H+UV[2]	0.015	2630.1	40.0	500,000	1/8	12	4.6	4.6
					50,000	0/8	<12	>3.6	
					5,000	0/8	<12	>2.6	
5	H+UV[2]	0.015	2654.2	40.0	500,000	7/8	146	3.5	3.4
					50,000	3/8	29	3.2	
					5,000	0/8	<12	>1.8	
6	H+UV[2]	0.015	2691.6	40.0	500,000	3/8	29	4.2	4.2
					50,000	0/8	<12	>3.6	
					5,000	0/8	<12	>2.6	
							Average		**4.1±0.28[3]**

[1] Filtered water homogenized after UV exposure
[2] Filtered water homogenized before UV exposure
[3] Mean square error generated by ANOVA

Table 4-7 shows the results of UV exposure experiments conducted on filtered water from run no. 5 at UV dose of 40 mJ/cm^2. The exposure procedures and conditions were similar to that followed in trials with 5 mJ/cm^2. The only exception was that the exposures were carried out in beakers instead of Petri dishes. The average log inactivation of filtered water samples that were homogenized before UV exposure was 4.1, whereas the average log inactivation of samples that were homogenized after UV exposure was 3.0. A single factor ANOVA analysis was performed in order to determine whether this difference was significant or not. The summary of ANOVA analysis is provided in Table B-2 (Appendix B). Despite having a difference of 1.1 log unit between the mean inactivation values of UV+H and H+UV samples, ANOVA revealed that this difference was not significant at the 95% confidence level (p = 0.069). However, the p value suggests that the difference was significant at the 93% confidence level. This analysis is inconclusive since the sample size was very small (n = 3). Sample size increases the power of ANOVA to detect small difference. In case phosphate buffered water, the average log inactivation of the oocysts at 40 mJ/cm^2 was >4.7. Therefore, the reduction in average log inactivation of the oocysts in filtered water compared to phosphate buffered water was >1.7.

4.4.3 Controls

The measured inactivation of the positive or zero-dose control samples is presented in Table 4-8. Inactivation in the positive controls that was significantly different than zero would suggest that handling, manipulation and storage of the oocysts during the experimental procedure, apart from UV exposure, was contributing to loss of infectivity. Furthermore, the inactivation of the positive controls would also indicate whether the homogenization step contributed to any loss (or increase) of infectivity. The log inactivation values in the positive controls ranged from -0.25 to 0.64 with an average of 0.18. The range of variation is can be attributed to the natural variation associated with the infectivity assay.

Negative controls were essentially carried out to evaluate the specificity of the foci detection method for detection of live and infectious oocysts. Also in the CC-FDM method, negative controls were used to detect sloughing of host cells from the chamber slide by comparing the monolayer of negative controls with infected monolayers. Infection was not detected in any of the negative control experiments carried out in this study (data not shown). There was also no evidence of sloughing in any of the dose-response or UV exposure trials which were used for establishing research objectives.

Table 4-8 Results from control trials carried out along with the UV exposure trials

Controls	Trial No	Type	Experimental suspension	d_0	P	d	$-\log(d/d_0)$
1	1	positive	buffered water	25	1/8	11	0.34
2	2	positive	buffered water	50	1/8	11	0.64
3	3,4	positive	buffered water	100	7/8	176	-0.25
5	5	positive	buffered water	100	5/8	64	0.19
7	6,7	positive	buffered water	100	4/8	45	0.35
9	8, 10	positive	buffered water	100	7/8	176	-0.25
11	9	positive	buffered water	100	3/8	31	0.5
12	4, 3, 1	positive	filtered water-3	100	5/8	57	0.2
14	6, 2, 5	positive	filtered water-3	100	6/8	84	0.1
16	3, 6, 1	positive	filtered water-4	100	5/8	57	0.2
18	2, 5, 4	positive	filtered water-4	100	7/8	146	-0.2
20	4, 2	positive	filtered water-5	100	4/8	41	0.4
22	6, 3, 5, 1	positive	filtered water-5	100	6/8	84	0.1

4.5 DIRECT MEASUREMENT OF OOCYTS AGGREGATION

In order to measure the degree of aggregation of oocysts, filtered and backwash water samples were passed through a series of polycarbonate track edged (PCTE) membrane filters. The filter sizes evaluated in this experiment were 10 μm, 5 μm, 2 μm and 0.8 μm. The number of oocysts retained in each filter was enumerated using hemacytometer count. A suspension of clean, particle free oocysts was also filtered through the same series of PCTE filters to determine the size distribution of monodispersed oocysts. The number of oocysts captured in each size filter was expressed as percentage of the total number of oocysts recovered using this method and is presented in Table E-1 (Appendix E).

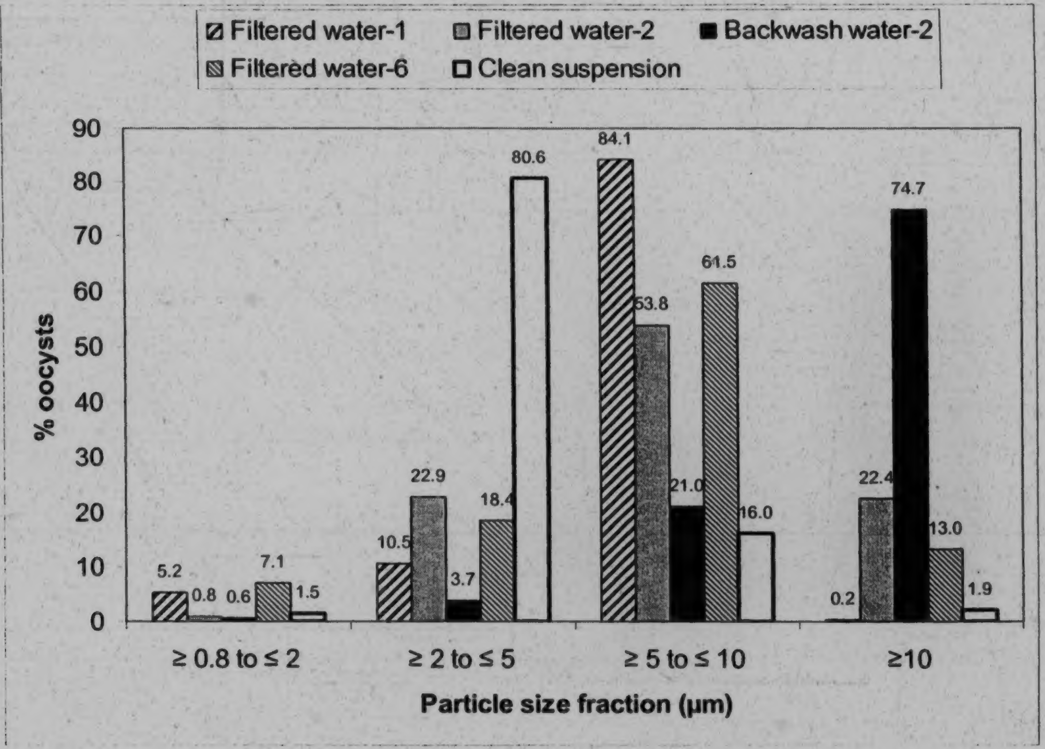


Figure 4.6 **Percent of total recovered oocyts retained on PCTE filters with different pore size**

Figure 4.6 shows percent of recovered oocyst captured in different size filter from each sample. *C. parvum* oocyst has a dimension of 3 to 6 µm (Belosivec et al., 2000) and theoretically most of the oocysts should be in the ≥2 to ≤5 and ≥5 to ≤10 µm size range. This was supported by the size distribution of clean oocysts suspension which shows that approximately 81% and 16%of the recovered oocysts were in the ≥2 to ≤5 and ≥5 to ≤10 size range respectively. However, a small fraction of the recovered oocysts (1.9%) in clean suspension was in the ≥10 size range which might have resulted from inadequate filtration of the PCTE filters. The general hypothesis is, oocysts that passed through a filter with specific pore size (i.e. 10 µm) and captured in a filter with smaller pore is likely to be associated with particles in that size range (≥ 5 ≤ 10). From Figure 4.5, it can be observed that for filtered water samples, the fraction of oocysts that was associated with particles ≥10 µm in size ranged from 0.2 to 22.4%. Similarly, the fraction of oocysts that was associated with particles ≥5 to ≤10 µm in size ranged from 53.8 to 84.1%. These observations are consistent with the research hypothesis that the oocysts that break through the filter media might be in aggregated state. For backwash water sample, a large fraction (74.7%) of the recovered oocysts was associated with particles with size ≥10 µm. This is quite predictable since the backwash water contains larger floc particles which were captured in the interstices of the filter media.

The overall recovery of oocysts for each sample ranged from 5.94 to 55.97%. The relatively low recovery on the PCTE filters may have been due to incomplete removal of particles from the filter surfaces or the presence of filter pore openings larger than the rated nominal pore size. The incomplete removal of oocysts from filter surfaces may have resulted from insufficient scraping and washing or loss of oocysts that were trapped in the pores Examination of PCTE membrane filter surfaces using scanning electron microscopy (SEM) revealed the presence of openings considerably larger than the nominal pore size due to imperfections in the track etching process.

Cake formation on the PCTE membrane filter can lead to a reduction of effective pore size on that filter. Such reduction may result in particles and oocysts being captured on filters of larger rated pore size. Examination of SEM images of the 5 and 10 µm size filters which were used for serial filtration of filtered water (Figures 4.7 to 4.9) provided little evidence of cake formation on the filter surfaces. Most of the pores appeared clear and unobstructed. This finding suggests that the effective cut-off size of the filters did not change during serial filtration.

4.6 MICROSCOPIC EXAMINATION OF OOCYSTS AGGREGATION

Two types of microscopic methods were used to examine the aggregation of oocysts in filtered water. The images captured from these microscopic methods are provided in the following sections

4.6.1 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used to examine the surface of the PCTE membrane filters used for serial filtration of water. The objective was to directly observe the aggregation of oocysts captured in different size filters. In addition, the degree of cake formation on PCTE filters was also examined. The washing and dehydration steps used in the preparation of samples prior to gold sputtering may create experimental artifacts which can alter the characteristics of oocyst aggregation on the filter surface. Nevertheless, SEM images provided a qualitative assessment of the state of aggregation of *C. parvum* oocyst in filtered drinking water.

Figure 4.7 to 4.9 shows the SEM micrographs of PCTE membrane filters of different pore size that were used to capture aggregated oocysts from filtered water. Additional images are provided in Appendix E. In most of the images, it was difficult to identify the oocysts. Oocyst-like particles were identified based on size and shape and are indicated by arrows in those images. Also, only oocyts that were attached to the surface of particles were visible. If oocysts were engulfed by the floc particles they would not be visible in the SEM images.

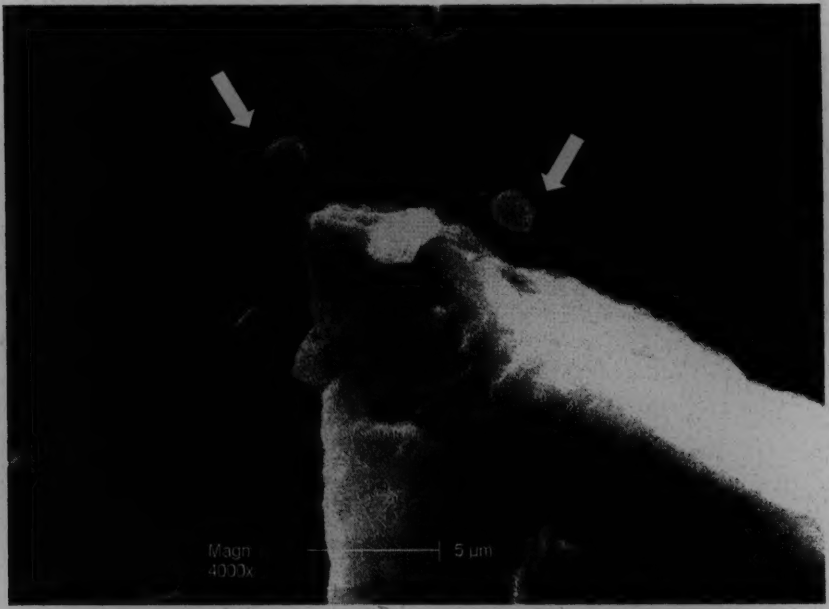


Figure 4.7 **SEM micrograph of a large floc particle surrounded by *C. parvum* oocyst-like particles which might have been detached from the surface of the floc particle. The pore size of the PCTE filter was 5 µm.**

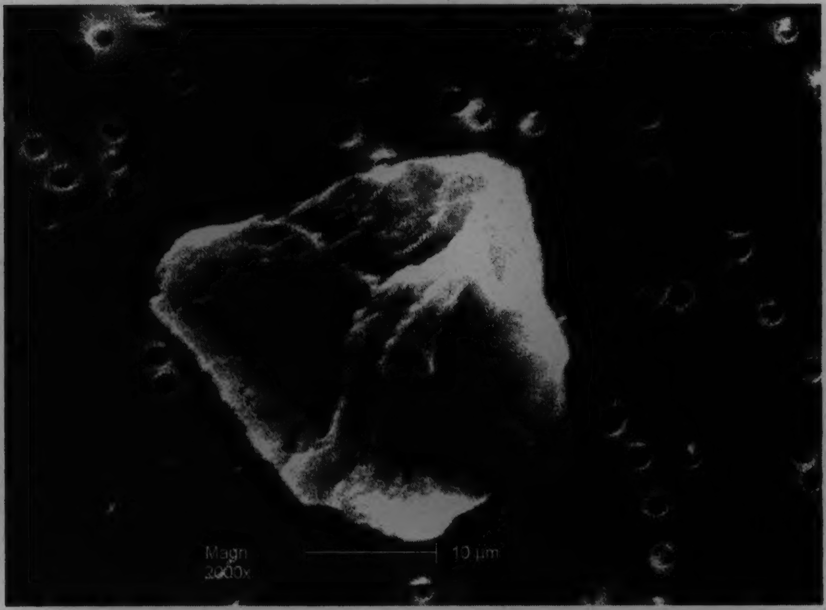


Figure 4.8 **SEM micrograph showing large floc particle (approximately 30 µm) captured in a 5 µm pore size PCTE filter**

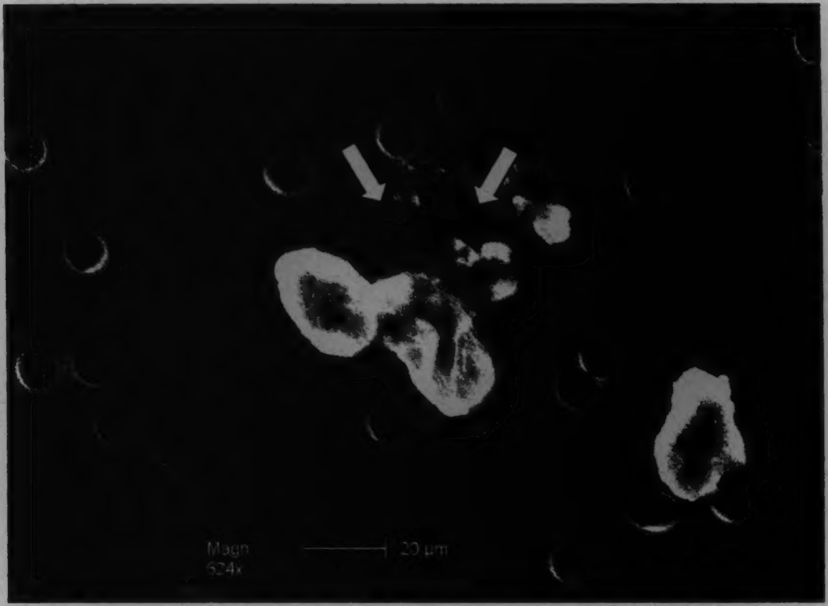


Figure 4.9 SEM micrograph showing oocyst-like particles attached to other particles captured on a the surface of a 10 µm pore size PCTE filter

4.6.2 Confocal Microscopy

In this method, oocysts captured from filtered water on the surfaces of the 0.8 µm PCTE filters were stained with Aqua-Glo™ antibody reagent and examined using a confocal microscope. Stained oocysts appeared apple green when viewed under fluorescence microscopy. The state of aggregation of the oocysts was observed by superimposing fluorescence image over the differential interface contrast (DIC) image of a particular field. Superimposing of the two images allowed visualization of particles and oocysts simultaneously and provided information about the state and nature of the oocyst aggregation in filtered water.

Figures 4.10 to 4.15 show the DIC-IFA images of C. *parvum* oocysts that were captured from filtered water. Additional images are provided in Appendix E. The oocysts were identified easily from their apple green fluorescence. Figure 4.10 shows that some of the oocysts in filtered water were associated with larger size particles. Figures 4.11 and 4.12 reveal that the oocysts in filtered water can be either attached to the surface of a floc particle or they can be engulfed by the floc particle. Evidence of oocysts associated with other oocysts in filtered water is shown in Figures 4.13 and 4.14.

The effect of homogenization on the state of aggregation of the oocysts was evaluated by examining the DIC-IFA image of oocysts captured from a homogenized filtered water samples (Figure 4.15). From figure 4.15, it can be observed that homogenization broke down the large floc particles into smaller size particles and thereby released the oocysts that were associated with the floc particles. Oocysts in the homogenized sample were more dispersed than those in the non homogenized sample (Figure 4.10). However, there were still some oocysts present in small aggregates. This suggests that homogenization could not disaggregate and disperse the oocysts completely.

Figure 4.10 DIC-IFA image of aggregated *C. parvum* oocysts captured from filtered water. Oocysts appear to be associated with large floc particles

Figure 4.11 DIC-IFA image of a floc particle captured from filtered water. *C. parvum* oocysts appear to be embedded on the floc particle surface.

Figure 4.12 DIC-IFA image of a floc particle captured from filtered water. *C. parvum* oocysts appear to be embedded within the floc particle

Figure 4.13 DIC-IFA image of a *C. parvum* oocyst clump captured from filtered water.

Figure 4.14 DIC-IFA image of a *C. parvum* oocyst clump captured from filtered water.

Figure 4.15 DIC-IFA image of *C. parvum* oocysts that were captured from a homogenized filtered water sample. Large floc particles were broken down into smaller size particles by the homogenization step

5.0 DISCUSSION

5.1 WATER TREATMENT PROECESS SIMULATION

One of the objectives of the study was to evaluate the effect of upstream treatment processes on UV inactivation of *C. parvum*. In order to accomplish that objective, water treatment processes were simulated at bench-scale. Untreated river water was spiked with *C. parvum* oocysts and was passed through that simulated process. Oocysts collected in the filtered water were exposed to UV irradiation using a collimated beam apparatus. The hypothesis was that the oocysts in filtered water might be in a aggregated state and that this can adversely affect the efficiency of UV disinfection. Therefore, it was extremely important to make sure that the state of aggregation of the oocysts in filtered was not disturbed by any experimental artifact. Concentration by centrifuge, membrane filtration or any other means of reducing the volume of the filtered water was avoided because these techniques could potentially alter the state of aggregation of the oocysts. The collimated beam apparatus was preferred for exposure experiments for two reasons. Firstly, the UV dose delivery in a collimated beam apparatus is well-defined and accurate. Secondly, collimated beam apparatus allows a better physical control of the experimental procedures and containment of samples. Since biohazard issues are involved while working with live *C. parvum* oocysts, controlling the experiments was extremely important.

Sub-optimal coagulation was intentionally adopted in this study in order to recover sufficient oocysts in filtered water required for determination of log inactivation following UV exposure. Since many water borne outbreaks of *C. parvum* were reported to be associated with suboptimal filter operation (Mackenzie et al., 1994; PHAC, 2001), it also provided an opportunity to observe the types and characteristics of oocyst aggregation that broke though the treatment barrier during periods of sub optimal operation and subsequently the effect of this aggregation on UV inactivation. From the observation of turbidity and particle count data of all the filtration runs, it can be concluded that turbidity was not a reliable indicator of oocysts breakthrough in filtered water. Particle counts, however, seemed to identify the oocysts breakthrough more accurately than turbidity.

5.2 HOMOGENIZATION

The UV dose requirement table developed by USEPA for log inactivation credit of C. *parvum* was based on the studies where oocysts suspended in either laboratory water or filtered water were exposed to UV irradiation. The oocysts were relatively mono dispersed in the test suspension and the possibility of aggregation of the oocysts generated by upstream treatment process was not taken into account. In this study a homogenizing step was used to disrupt the aggregates and disperse the oocysts before or after UV exposure. It should be noted that the homogenization step was purely an experimental technique used to observe the effects of aggregation on UV disinfection. The samples which were homogenized before UV exposure were assumed to be equivalent to the experiments in which the oocysts are spiked into clean water. On the other hand, the samples which were homogenized after UV exposure represent the feed water of a UV reactor in a water treatment plant under actual field conditions. Homogenization after UV exposure was necessary for the infectivity assay. No negative impact of the homogenization step on the infectivity of the C. *parvum* oocysts was observed. Confocal microscopic image of oocysts captured from homogenized filtered water revealed that the homogenization step could disperse the oocysts (Figure 4.15) to some extent. The homogenization step used in this study may be difficult to implement in water treatment plants because of large volume of water and high energy required to achieve same level of mixing.

5.3 INFECTIVITY ASSAY

A cell culture based infectivity assay was used in this study for determination of log inactivation following UV exposure. Cell culture based infectivity assays were reported to demonstrate equivalency to mouse infectivity assays and are considered a practical alternative for measuring the infectivity of C. *parvum* oocysts (Rochelle et al., 2002a; Slifko et al., 2002). Detection of infectious oocysts in cell monolayer was accomplished by using the foci detection method. In this method, the cell monolayers were stained with Sporo-Glo™ antibody and infectious life stages were detected using epi-fluorescence microscopy. Positive infection was determined by the presence of visible sporozoite invasion that produced an infectious focus, and clustering, a result of secondary infection. Oocysts that were inactivated by UV irradiation underwent excystation and invaded HCT-8 cells, but they could not go through the secondary infection stages resulting in creating discrete pin points of invasion (Figure 3.5). Slifko et al. (1999) observed that oocysts that were treated with pulse broad spectrum light could not proceed past the sporozoite invasion stage after 48h of incubation and thereby they could not form clusters of foci. Detection of clusters of infection was extremely important to differentiate between infectious and non-infectious oocysts. Foci detection method was preferred over molecular methods (i.e. PCR) because the clustering of foci provides a clear indication that reproduction has occurred. Molecular assays may detect the DNA or RNA produced in the invasion stage and may overestimate the total number of infectious oocyst following UV exposure (Slifko et al., 1999). However, Rochelle and Leon (2001) reported that targeting mRNA for detection of infectious oocysts can reduce the possibility of false positive detection since mRNA degrades if the oocysts are incapable of reproduction.

Before inoculation of the cell monolayers, the concentrated samples were pretreated with 10% bleach in order to induce oocyst excystation and to prevent contamination of the cells by bacteria and fungi. Whether this pretreatment technique has any synergistic effect in UV disinfection studies is yet to be investigated. Di Giovanni et al. (1999) reported that the final step of USEPA method 1623, which involves dissociation of oocysts from immunomagnetic beads in 0.1 M HCL reduces the infectivity of oocysts in cell culture. The investigators recommended using acidified hanks balanced salt solution (AHBSS) combined with 1% trypsin for oocyst pretreatment. In the present study, preteating with AHBSS-1% trypsin in place of bleach did not prevent contamination in the cell monolayers (data not shown) and hence this technique was not adopted.

Infection of cells by C. *parvum* may induce apoptotic cell death in the host monolayer. Widespread cell death in infected monolayers may result in sloughing of cells from growth chamber, the elimination of C. *parvum* receptive cells from the monolayers, and may be partly responsible for the lack of continuous auto reinfection cycle in cell culture (Rochelle and Leon, 2001). In this study, cell monolayers were routinely monitored during the incubation period following inoculation with oocysts in order to detect sloughing of cells from the chamber slides. In addition, infected monolayers were compared with non-infected monolayers (inoculated with heat inactivated oocysts) during microscopic examination to make sure that the infected monolayers were intact. However, it was observed that the age of the cells, number of passages and cell storage technique may contribute to cell detachment from the chamber slides.

In the present study, same cell culture technique was consistently used for all the samples (phosphate buffered water, UV+H and H+UV) for determining the loss in oocyst infectivity following UV exposure. In this way, the effect cell culture infectivity assay (i.e. pretreatment with 10% bleach, cell detachment from the monolayers etc) were assumed to be identical for all the samples. Besides, the UV dose-response of C. *parvum* obtained in this study was comparable with past studies using animal infectivity models (Figure 4.5).

5.4 UV EXPOSURE EXPERIMENTS

The interpretation of results in this study in terms of UV disinfection performance will provide useful information about the effects of aggregation on UV inactivation of C. *parvum* in filtered drinking water. In this study, the difference in log inactivation of C. *parvum* oocysts between filtered water samples that were homogenized before UV exposure (H+UV) and samples that were homogenized after UV exposure (UV+H) was evaluated at two different UV dose levels; 5 and 40 mJ/cm^2. As mentioned earlier, some of the oocysts in UV+H samples were presumed to be in aggregated state whereas oocysts in H+UV samples were assumed to be in mono dispersed state. At a dose of 5 mJ/cm^2, the average difference in log inactivation between UV+H and H+UV sample was 0.8 and this difference was statistically significant at the 95% confidence level (n = 6, p = 0.02). This suggests that there was a significant reduction in UV inactivation of *C. parvum* oocysts that broke through the filter media compared to the oocysts that were mono dispersed. At 40 mJ/cm^2 the average difference in log inactivation between UV+H and H+UV samples was 1.1 but this difference was not found to be statistically significant at 95% confidence level (n = 3, p = 0.069). However, the p value suggests that difference is marginally insignificant and since the number of observation was small, the statistical analysis is inconclusive. Nevertheless, the results at 40 mJ/cm^2 suggests that there is a possibility that a fraction of oocysts in filtered drinking water might escape the UV disinfection process even at higher applied dose.

The findings of this study conflict with the findings of some earlier studies which reported that the UV inactivation of MS2 coliphage was independent of turbidity provided that the absorbance of the water was taken into account in the determination of UV dose (Batch et al., 2004; Passantino et al., 2004). One possible explanation for this difference in findings is that the microorganisms in those studies were spiked into the test water with different turbidity levels. Hence, the microorganisms had very liitle opportunity to interact with particles and to aggregates. In the present study, *C. parvum* oocysts were spiked into raw water and the raw water was coagulated, flocculated, settled and filtered prior to UV exposure. These processes encouraged the aggregation of the oocysts in filtered water which in turns affected the efficiency of UV inactivation. Amoah et al. (2005) reported that the UV inactivation of *C. parvum* was not significantly affected (p = 0.06) when the turbidity of water was increased from 0.25 to 10 NTU. However the authors observed a modest reduction in UV inactivation of *C. parvum* (0.8 log) when the turbidity was increased from 0.25 to 20 NTU. Interestingly, in this study, similar reduction in UV inactivation of the *C. parvum* oocysts was observed at much lower filtered water turbidity levels. The average turbidity of the filtered water samples that were exposed to UV dose of 5 and 40 mJ/cm^2 were 1.46 and 3.12 NTU respectively. This suggests that turbidity is not a very reliable indicator of oocysts aggregation in filtered water. The findings of this study aggres well with the study conducted by

Uvbiama and Craik (2005) where the investigators observed statistically significant reduction in UV inactivation of *Bacillus subtilis* in filtered water samples that were homogenized after UV exposure compared to the samples that were homogenized before UV exposure.

5.5 SERIAL FILTRATION AND MICROSCOPY

The results obtained from the serial filtration experiments suggested that about 0.2 to 22.4% and 53.8 to 84.1% of the total recovered oocysts in filtered water were in aggregates of greater or equal to 10 μm and 5 to 10 μm size range respectively. Given that most of the oocysts used in this study were in the size range of 2-5 μm (as observed from the serial filtration of clean oocyst suspension), these observations support the hypothesis that some oocysts in filtered drinking are likely to be in an aggregated state. However, the results from the serial filtration experiments should be interpreted cautiously because the recovery of oocysts from filtered water was low and showed considerable variation. It should also be noted here that the oocysts used for serial filtration and microscopic experiments were formalin fixed. Emelko et al. (2003) showed that the formalin fixed oocysts are reliable surrogates of viable oocysts in filtration experiments. Therefore, it can be assumed that observations of the serial filtration and microscopic experiments will hold true for live oocysts.

Microscopic examination of the aggregated oocysts in filtered water revealed the existence of three types of aggregation. These are, (i) Attachment of oocysts to the surface of the particles (Figures 4.7, 4.10 to 4.12), (ii) Association of oocysts with other oocysts (Figures 4.13, 4.14) and (iii) Enmeshment or complete covering of oocysts into the floc particles (Figures 4.9, 4.11, 4.12). In conclusion, both serial filtration and microscopic examination were consistent with the hypothesis of the study and with the observed reduction in UV inactivation of *C. parvum*.

In this study, the effect of upstream treatment processes on the aggregation and UV inactivation of *C. parvum* oocysts was observed under a limited set of conditions. It might be worthwhile to vary some of the experimental conditions, i.e. type of raw water, type of coagulant and coagulant aid, coagulant dose etc. and observe their effect on the results. The turbidity of the filtered water in this study was higher than the typical regulatory limit (i.e. 0.3 NTU). Therefore, it remains to be seen whether similar reduction in UV inactivation of C. *parvum* will be observed in filtered water with turbidity <0.3 NTU.

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The objective of this study was to observe the state of aggregation of C. *parvum* oocyst in filtered water and to evaluate the effect of this aggregation on the performance of UV disinfection. The conclusions that could be reached from the findings of the study are as follows:

- Some of the C. *parvum* oocysts that broke through the filter media during periods of sub-optimal coagulation were in an aggregated state. This aggregation was evident in serial filtration experiments which demonstrated that a fraction of the oocysts in filtered water were associated with other particles or oocysts. Microscopic examination of the aggregated oocysts provided further evidence of this aggregation and also showed the possible types of aggregation in filtered water.

- The UV inactivation of C. *parvum* oocysts was reduced by 0.8 and 1.1 log-unit at 5 and 40 mJ/cm^2 respectively as a result of the aggregation. This reduction in UV inactivation at 5 mJ/cm^2 was statistically significant at the 95% confidence level ($p = 0.022$, n = 6). The reduction in UV inactivation of the oocysts at 40 mJ/cm^2 was however, statistically insignificant at the 95% confidence level ($p = 0.069$, n = 3) but was significant at the 93% confidence level. Nevertheless, these results suggest that the UV inactivation of C. *parvum* oocysts in poorly filtered water might be overestimated if the effect of oocyst aggregation is not taken into consideration.

6.2 RECOMMENDATIONS

The recommendations of this study are as follows:

- Since UV inactivation of C. *parvum* has been shown to be affected by the presence of aggregated oocysts in filtered water, UV dose/inactivation credit should be conservative to account for possibility of aggregation.

- Sub-optimal coagulation resulted in break through of aggregated oocysts in filtered water. Therefore, optimal operation of the conventional water treatment processes must be ensured even with UV disinfection in place.

- Turbidity measurements during filtration runs could not identify the oocyst breakthrough in filtered water. Particle count was a better indicator in this respect. However, both turbidity and particle count should be used cautiously as indicators of oocysts breakthrough in filtered water.

- The findings of this study were based on a bench-scale experimental set up. These findings should be confirmed in a pilot-scale water treatment facility.

- For future studies, the state of oocyst aggregation in low turbidity filtered water (i.e. ≤0.3 NTU) and its effect on UV disinfection could be investigated. In addition, different experimental conditions i.e. coagulant type, microorganism type could be varied and their effect on UV disinfected could be observed.

REFERENCES

Amoah, K., S. Craik, D. W. Smith and M. Belosevic (2005). Inactivation of Cryptosporidium oocysts and Giardia cysts by ultraviolet light in the presence of natural particulate matter. *Journal of Water Supply Research and Technology-Aqua.* 54(3): 165-178.

Batch, L. E., C. R. Schulz and K. G. Linden (2004). Evaluting water qualify effects on UV disinfection of MS2 coliphage. *Journal American Water Works Association.* 96(7): 75-87.

Belosevic, M., N. Neumann, F., L. L. Gyurek and G. R. Finch (2000). *Cryptosporidium Parvum Viability Assay.* AWWA Research Foundation and the American Water Works Association.

Belosivec, M., N. Neumann, F., L. L. Gyurek and G. R. Finch (2000). *Cryptosporidium Parvum Viability Assay.* AWWA Research Foundation and the American Water Works Association.

Betancourt, W. Q. and J. B. Rose (2004). Drinking water treatment processes for removal of Cryptosporidium and Giardia. *Veterinary Parasitology.* 126(1-2): 219-234.

Bolton, J. R. (2001). *Ultraviolet Applications Handbook.* Bolton Photosciences Inc. Edmonton

Bolton, J. R. and K. G. Linden (2003). Standardization of methods for fluence (UV dose) determination in bench-scale UV experiments. *Journal of Environmental Engineering-Asce.* 129(3): 209-215.

Bolton, J. R. and M. I. Stefan (2002). Fundamental photochemical approach to the concepts of fluence (UV dose) and electrical energy efficiency in photochemical degradation reactions. *Research on Chemical Intermediates.* 28(7-9): 857-870.

Bukhari, Z., T. M. Hargy, J. R. Bolton, B. Dussert and J. L. Clancy (1999). Medium-pressure UV for oocyst inactivation. *Journal American Water Works Association.* 91(3): 86-94.

Bukhari, Z., D. M. Holt, M. W. Ware and F. W. Schaefer (2005). 'Blind' trials evaluating *in vitro* infectivity of *Cryptosporidium parvum* using cell culture-Immunofluorescence (IFA). Third International Congress on Ultraviolet Technologies, Whistler, BC, International Ultraviolet Association.

Bukhari, Z. and M. LeChevallier (2003). Assessing UV reactor performance for treatment of finished water. *Water Science and Technology*. 47(3): 179-184.

Butkus, M. A., J. T. Bays and M. P. Labare (2003). Influence of surface characteristics on the stability of Cryptosporidium parvum oocysts. *Applied and Environmental Microbiology*. 69(7): 3819-3825.

Caccio, S. M., R. C. A. Thompson, J. McLauchlin and H. V. Smith (2005). Unravelling Cryptosporidium and Giardia epidemiology. *Trends in Parasitology*. 21(9): 430-437.

Christensen, J. and K. G. Linden (2003). How particles affect UV light in the UV disinfection of unfiltered drinking water. *Journal American Water Works Association*. 95(4): 179-189.

Clancy, J. L., Z. Bukhari, T. M. Hargy, J. R. Bolton, B. W. Dussert and M. M. Marshall (2000). Using UV to inactivate Cryptosporidium. *Journal American Water Works Association*. 92(9): 97-104.

Clancy, J. L., T. M. Hargy, M. M. Marshall and J. E. Dyksen (1998). UV Light Inactivation of *Cryptosporidium* Oocysts. *Journal American Water Works Association*. 90(9): 92-102.

Considine, R. F., D. R. Dixon and C. J. Drummond (2002). Oocysts of Cryptosporidium parvum and model sand surfaces in aqueous solutions: an atomic force microscope (AFM) study. *Water Research*. 36(14): 3421-3428.

Cotton, C. and L. Passantino (2005). Regulations in the United States: requirements and guidance for ultraviolet disinfection of drinking water. *Journal of Environmental Engineering and Science*. 4: S57-S63.

Craik, S. A., D. Weldon, G. R. Finch, J. R. Bolton and M. Belosevic (2001). Inactivation of Cryptosporidium parvum oocysts using medium- and low-pressure ultraviolet radiation. *Water Research*. 35(6): 1387-1398.

Craun, G. F., S. A. Hubbs, F. Frost, R. L. Calderon and S. H. Via (1998). Waterborne outbreaks of cryptosporidiosis. *Journal American Water Works Association*. 90(9): 81-91.

Current, W. L. and L. S. Garcia (1991a). Cryptosporidiosis. *Clinics in Laboratory Medicine*. 11(4): 873-897.

Current, W. L. and L. S. Garcia (1991b). Cryptosporidiosis. *Clinical Microbiology Reviews.* 4(3): 325-358.

Current, W. L., N. C. Reese, J. V. Ernst, W. S. Bailey, M. B. Heyman and W. M. Weinstein (1983). Human Cryptosporidiosis in Immunocompetent and Immunodeficient Persons - Studies of an Outbreak and Experimental Transmission. *New England Journal of Medicine*. 308(21): 1252-1257.

Di Giovanni, G. D., F. H. Hashemi, N. J. Shaw, F. A. Abrams, M. W. LeChevallier and M. Abbaszadegan (1999). Detection of infectious Cryptosporidium parvum oocysts in surface and filter backwash water samples by immunomagnetic separation and integrated cell culture-PCR. *Applied and Environmental Microbiology.* 65(8): 3427-3432.

Drozd, C. and J. Schwartzbrod (1996). Hydrophobic and electrostatic cell surface properties of Cryptosporidium parvum. *Applied and Environmental Microbiology.* 62(4): 1227-1232.

Dugan, N. R., K. R. Fox, J. H. Owens and R. J. Miltner (2001). Controlling Cryptosporidium oocysts using conventional treatment. *Journal American Water Works Association*. 93(12): 64-76.

Emelko, M. B. (2003). Removal of viable and inactivated Cryptosporidium by dual- and tri-media filtration. *Water Research.* 37(12): 2998-3008.

Emelko, M. B., P. M. Huck and B. M. Coffey (2005). A review of Cryptosporidium removal by granular media filtration. *Journal American Water Works Association.* 97(12): 101-+.

Emerick, R. W., F. J. Loge, T. Ginn and J. L. Darby (2000). Modeling the inactivation of particle-associated coliform bacteria. *Water Environment Research.* 72(4): 432-438.

Emerick, R. W., F. J. Loge, D. Thompson and J. L. Darby (1999). Factors influencing ultraviolet disinfection performance part II: Association of coliform bacteria with wastewater particles. *Water Environment Research.* 71(6): 1178-1187.

Fayer, R. and R. G. Leek (1984). The Effects of Reducing Conditions, Medium, Ph, Temperature, and Time on Invitro Excystation of Cryptosporidium. *Journal of Protozoology.* 31(4): 567-569.

Fayer, R., U. Morgan and S. J. Upton (2000). Epidemiology of Cryptosporidium: transmission, detection and identification. *International Journal for Parasitology.* 30(12-13): 1305-1322.

Glasgow, G. D. E. and A. D. Wheatley (2001). Particle ripening, removal and breakthrough in drinking-water filtration: Implications for the capture of Cryptosporidium and Giardia cysts. *Journal of the Chartered Institution of Water and Environmental Management*. 15(3): 193-197.

Gyürék, L. L., G. R. Finch and M. Belosevic (1997). Modeling Chlorine Inactivation Requirements of *Cryptosporidium parvum* Oocysts. *Journal of Environmental Engineering*. 123(9): 865-875.

Harm, W. (1980). *Biological Effects of Ultraviolet Radiation*. Cambridge University Press. Cambridge

Harrington, G. W., I. Xagoraraki, P. Assavasilavasukul and J. H. Standridge (2003). Effect of filtration conditions on removal of emerging waterborne pathogens. *Journal American Water Works Association*. 95(12): 95-104.

Huck, P. M., B. M. Coffey, M. B. Emelko, D. D. Maurizio, R. M. Slawson, W. B. Anderson, J. Van den Oever, I. P. Douglas and C. R. O'Melia (2002). Effects of filter operation on Cryptosporidium removal. *Journal American Water Works Association*. 94(6): 97-111.

Johnson, A. M., K. Linden, K. M. Ciociola, R. De Leon, G. Widmer and P. A. Rochelle (2005). UV inactivation of Cryptosporidium hominis as measured in cell culture. *Applied and Environmental Microbiology*. 71(5): 2800-2802.

Jolis, D., C. Lam and P. Pitt (2001). Particle effects on ultraviolet disinfection of coliform bacteria in recycled water. *Water Environment Research*. 73(2): 233-236.

Karaman, M. E., R. M. Pashley, H. Bustamante and S. R. Shanker (1999). Microelectrophoresis of Cryptosporidium parvum oocysts in aqueous solutions of inorganic and surfactant cations. *Colloids and Surfaces a-Physicochemical and Engineering Aspects*. 146(1-3): 217-225.

Korich, D. G., J. R. Mead, M. S. Madore, N. A. Sinclair and C. R. Sterling (1990). Effects of Ozone, Chlorine Dioxide, Chlorine, and Monochloramine on Cryptosporidium-Parvum Oocyst Viability. *Applied and Environmental Microbiology*. 56(5): 1423-1428.

LeChevallier, M. W., G. D. Di Giovanni, J. L. Clancy, Z. Bukhari, S. Bukhari, J. S. Rosen, J. Sobrinho and M. M. Frey (2003). Comparison of method 1623 and cell culture-PCR for detection of Cryptosporidium spp. in source waters. *Applied and Environmental Microbiology*. 69(2): 971-979.

Lechevallier, M. W. and W. D. Norton (1992). Examining Relationships between Particle Counts and Giardia, Cryptosporidium, and Turbidity. *Journal American Water Works Association.* 84(12): 54-60.

Lechevallier, M. W., W. D. Norton and R. G. Lee (1991). Occurrence of Giardia and Cryptosporidium Spp in Surface-Water Supplies. *Applied and Environmental Microbiology.* 57(9): 2610-2616.

Levine, N. D. (1985). Phylum II. Apicomplexa Levine. An Illustrated guide to the Protozoa. J. L. John, H. H. Seymour, E. C. Bovee., Lawrence, K. S. : Society of Protozoologists: pp. 322 - 374.

Loge, F. J., R. W. Emerick, D. E. Thompson, D. C. Nelson and J. L. Darby (1999). Factors influencing ultraviolet disinfection performance part I: Light penetration to wastewater particles. *Water Environment Research.* 71(3): 377-381.

Lorenzolorenzo, M. J., M. E. Aresmazas, I. V. M. Dematurana and D. Duranoreiro (1993). Effect of Ultraviolet Disinfection of Drinking-Water on the Viability of Cryptosporidium-Parvum Oocysts. *Journal of Parasitology.* 79(1): 67-70.

Mackenzie, W. R., N. J. Hoxie, M. E. Proctor, M. S. Gradus, K. A. Blair, D. E. Peterson, J. J. Kazmierczak, D. G. Addiss, K. R. Fox, J. B. Rose and J. P. Davis (1994). A Massive Outbreak in Milwaukee of Cryptosporidium Infection Transmitted through the Public Water-Supply. *New England Journal of Medicine.* 331(15): 1035-1035.

Marshall, M. M., D. Naumovitz, Y. Ortega and C. R. Sterling (1997). Waterborne protozoan pathogens. *Clinical Microbiology Reviews.* 10(1): 67-&.

Martins, C. A. P. and R. L. Guerrant (1995). Cryptosporidium and Cryptosporidiosis. *Parasitology Today.* 11(11): 434-436.

Masschelein, W. J. (2002). *Ultraviolet Light in Water and Wastewater Sanitation*. Lewis Publishers.

Mofidi, A. A., H. Baribeau, P. A. Rochelle, R. De Leon, B. M. Coffey and J. F. Green (2001). Disinfection of Cryptosporidium parvum with polychromatic UV light. *Journal American Water Works Association.* 93(6): 95-109.

Montgomery, D. C. (2001). *Design and Analysis of Experiments*. New York

Morita, S., A. Namikoshi, T. Hirata, K. Oguma, H. Katayama, S. Ohgaki, N. Motoyama and M. Fujiwara (2002). Efficacy of UV irradiation in inactivating Cryptosporidium parvum oocysts. *Appl Environ Microbiol.* 68(11): 5387-93.

Neumann, N. F., L. L. Gyurek, L. Gammie, G. R. Finch and M. Belosevic (2000). Comparison of Animal Infectivity and Nucleic Acid Staining for Assessment of Cryptosporidium parvum Viability in Water. *Appl. Environ. Microbiol.* 66(1): 406-412.

Nieminski, E. C. and J. E. Ongerth (1995). Removing Giardia and Cryptosporidium by Conventional Treatment and Direct-Filtration. *Journal American Water Works Association.* 87(9): 96-106.

O' Donoghue, P. J. (1995). Cryptosporidium and Cryptosporidiosis in Man and Animals. *International Journal for Parasitology.* 25(2): 139-195.

Ongerth, J. E. and J. P. Pecoraro (1995). Removing Cryptosporidium Using Multimedia Filters. *Journal American Water Works Association.* 87(12): 83-89.

Ongerth, J. E. and J. P. Pecoraro (1996). Electrophoretic mobility of Cryptosporidium oocysts and Giardia cysts. *Journal of Environmental Engineering-Asce.* 122(3): 228-231.

Parker, J. A. and J. L. Darby (1995). Particle-Associated Coliform in Secondary Effluents - Shielding from Ultraviolet-Light Disinfection. *Water Environment Research.* 67(7): 1065-1075.

Passantino, L., J. Malley, M. Knudson, R. Ward and J. Kim (2004). Effect of low turbidity and algae on UV disinfection performance. *Journal American Water Works Association.* 96(6): 128-137.

PHAC (2001). Waterborne Cryptosporidiosis Outbreak, North Battleford, Saskatchewan, Spring 2001. *Canada Communicable Disease Report.* 27(22)

Qualls, R. G., M. P. Flynn and J. D. Johnson (1983). The Role of Suspended Particles in Ultraviolet Disinfection. *Journal Water Pollution Control Federation.* 55(10): 1280-1285.

Qualls, R. G., S. F. Ossoff, J. C. H. Chang, M. H. Dorfman, C. M. Dumais, D. C. Lobe and J. D. Johnson (1985). Factors Controlling Sensitivity in Ultraviolet Disinfection of Secondary Effluents. *Journal Water Pollution Control Federation.* 57(10): 1006-1011.

Quintero-Betancourt, W., E. R. Peele and J. B. Rose (2002). Cryptosporidium parvum and Cyclospora cayetanensis: a review of laboratory methods for detection of these waterborne parasites. *Journal of Microbiological Methods.* 49(3): 209-224.

Reduker, D. W. and C. A. Speer (1985). Factors Influencing Excystation in Cryptosporidium Oocysts from Cattle. *Journal of Parasitology.* 71(1): 112-115.

Reduker, D. W., C. A. Speer and J. A. Blixt (1985). Ultrastructure of Cryptosporidium-Parvum Oocysts and Excysting Sporozoites as Revealed by High-Resolution Scanning Electron-Microscopy. *Journal of Protozoology.* 32(4): 708-711.

Robertson, L. J., A. T. Campbell and H. V. Smith (1992). Survival of Cryptosporidium-Parvum Oocysts under Various Environmental Pressures. *Applied and Environmental Microbiology.* 58(11): 3494-3500.

Rochelle, P. A., R. De Leon, A. Johnson, M. H. Stewart and R. L. Wolfe (1999). Evaluation of immunomagnetic separation for recovery of infectious Cryptosporidium parvum oocysts from environmental samples. *Appl Environ Microbiol.* 65(2): 841-5.

Rochelle, P. A., D. Fallar, M. M. Marshall, B. A. Montelone, S. J. Upton and K. Woods (2004). Irreversible UV inactivation of Cryptosporidium spp. despite the presence of UV repair genes. *Journal of Eukaryotic Microbiology.* 51(5): 553-562.

Rochelle, P. A., D. M. Ferguson, T. J. Handojo, R. De Leon, M. H. Stewart and R. L. Wolfe (1996). Development of a rapid detection procedure for Cryptosporidium, using in vitro cell culture combined with PCR. *J Eukaryot Microbiol.* 43(5): 72S.

Rochelle, P. A., D. M. Ferguson, T. J. Handojo, R. De Leon, M. H. Stewart and R. L. Wolfe (1997). An Assay Combining Cell Culture with Reverse Transcriptase PCR to Detect and Determine the Infectivity of Waterborne *Cryptosporidium parvum. Applied and Environmental Microbiology.* 63(5): 2029-2037.

Rochelle, P. A. and R. D. Leon (2001). A review of methods for assessing the infectivity of Cryptosporidium parvum using in-vitro cell culture. *Special publications of the royal society of chemistry.* 265: 88-95.

Rochelle, P. A., M. Marchall, J. R. Mead, A. M. Johnson, D. G. Korich, J. S. Rosen and R. De Leon (2002a). Comparison of In Vitro Cell Culture and a Mouse Assay for Measuring Infectivity of *Cryptosporidium parvum. Applied and Environmental Microbiology.* 68: 3809-3817.

Rochelle, P. A., M. M. Marshall, J. R. Mead, A. M. Johnson, D. G. Korich, J. S. Rosen and R. De Leon (2002b). Comparison of in vitro cell culture and a mouse assay for measuring infectivity of Cryptosporidium parvum. *Applied and Environmental Microbiology*. 68(8): 3809-3817.

Schuster, C. J., A. G. Ellis, W. J. Robertson, D. E. Charron, J. J. Aramini, B. J. Marshall and D. T. Medeiros (2005). Infectious disease outbreaks related to drinking water in Canada, 1974-2001. *Canadian Journal of Public Health-Revue Canadienne De Sante Publique*. 96(4): 254-258.

Searcy, K. E., A. I. Packman, E. R. Atwill and T. Harter (2005). Association of Cryptosporidium parvum with suspended particles: Impact on oocyst sedimentation. *Applied and Environmental Microbiology*. 71(2): 1072-1078.

Seber, G. A. F. and C. J. Wild (1989). *Nonlinear Regression*. John Wiley & Sons. New York

Shin, G. A., K. G. Linden, M. J. Arrowood and M. D. Sobsey (2001). Low-pressure UV inactivation and DNA repair potential of Cryptosporidium parvum oocysts. *Applied and Environmental Microbiology*. 67(7): 3029-3032.

Slifko, T. R., D. Friedman, J. B. Rose and W. Jakubowski (1997a). An Invitro Method for Detecting Infectious *Cryptosporidium* Oocysts with Cell Culture. *Applied and Environmental Microbiology*. 63(9): 3669-3674.

Slifko, T. R., D. E. Friedman, J. B. Rose, S. J. Upton and W. Jakubowski (1997b). Unique cultural methods used to detect viable Cryptosporidium parvum oocysts in environmental samples. *Water Science and Technology*. 35(11-12): 363-368.

Slifko, T. R., D. E. Huffman, B. Dussert, J. H. Owens, W. Jakubowski, C. N. Haas and J. B. Rose (2002). Comparison of tissue culture and animal models for assessment of Cryptospridium parvum infection. *Experimental Parasitology*. 101(2-3): 97-106.

Slifko, T. R., D. E. Huffman and J. B. Rose (1999). A most-probable-number assay for enumeration of infectious Cryptosporidium parvum oocysts. *Applied and Environmental Microbiology*. 65(9): 3936-3941.

Smith, H. V., R. A. B. Nichols and A. M. Grimason (2005). Cryptiosporidium excystation and invasion: getting to the guts of the matter. *Trends in Parasitology*. 21(3): 133-142.

Templeton, M. R., R. C. Andrews and R. Hofmann (2005). Inactivation of particle-associated viral surrogates by ultraviolet light. *Water Research*. 39(15): 3487-3500.

Upton, S. J., M. Tilley and D. B. Brillhart (1994). Comparative Development of Cryptosporidium-Parvum (Apicomplexa) in 11 Continuous Host-Cell Lines. *Fems Microbiology Letters*. 118(3): 233-236.

USEPA (1989). Surface Water Treatment Rule. Office of Water, Washington.Office of Water, Washington

USEPA (1999). Alternative Disinfectant and Oxidants Guidance Mannual. *EPA 815-R-99-014*. Office of Water, Washington.Office of Water, Washington

USEPA (2000). National primary drinking water regulations: ground water rule; proposed rules. *Federal Register*. **65**(91): 30194

USEPA (2001). Method 1623: Cryptosporidium and Giardia in Water by Filtration/IMS/FA. *EPA-821-R-01-025*. Office of Water, Washington.Office of Water, Washington

USEPA (2003). Ultraviolet disinfection guidance manual. *EPA 815-D-03-007*. Office of Water, Washington.Office of Water, Washington

USEPA (2006). National Primary Drinking Water Regulations: Long Term 2 Enhanced Surface Water Treatment Rule. *Federal Register*. **71**(3): 654

Uvbiama, D. R. and S. A. Craik (2005). The effect of upstream treatment processes on uv inactivation of microorganisms in filtered drinking water. Third International Congress on Ultraviolet Technologies, Whistler, BC, International Ultraviolet Association.

Widmer, G., D. Akiyoshi, M. A. Buckholt, X. Feng, S. M. Rich, K. M. Deary, C. A. Bowman, P. Xu, Y. Wang, X. Wang, G. A. Buck and S. Tzipori (2000). Animal propagation and genomic survey of a genotype 1 isolate of Cryptosporidium parvum. *Molecular and Biochemical Parasitology*. 108(2): 187-197.

Widmer, G., E. A. Orbacz and S. Tzipori (1999). beta-tubulin mRNA as a marker of Cryptosporidium parvum oocyst viability. *Applied and Environmental Microbiology*. 65(4): 1584-1588.

Wiedenmann, A., P. Kruger and K. Botzenhart (1998). PCR detection of Cryptosporidium parvum in environmental samples - a review of published protocols and current developments. *Journal of Industrial Microbiology & Biotechnology*. 21(3): 150-166.

Woods, K. M., M. V. Nesterenko and S. J. Upton (1995). Development of a Microtitre Elisa to Quantify Development of Cryptosporidium-Parvum in-Vitro. *Fems Microbiology Letters.* 128(1): 89-93.

Xagoraraki, I. and G. W. Harrington (2004). Zeta potential, dissolved organic carbon, and removal of Cryptosporidium oocysts by coagulation and sedimentation. *Journal of Environmental Engineering-ASCE.* 130(12): 1424-1432.

Yu, J. R., S. D. Choi and Y. W. Kim (2000). In vitro infection of Cryptosporidium parvum to four different cell lines. *Korean J Parasitol.* 38(2): 59-64.

Zimmer, J. L., R. M. Slawson and P. M. Huck (2003). Inactivation and potential repair of Cryptosporidium parvum following low- and medium-pressure ultraviolet irradiation. *Water Research.* 37(14): 3517-3523.

APPENDIX A INFORMATION FROM LOGISTIC DOSE RESPONSE EXPERIMENTS OF *CRYPTOSPORIDIUM PARVUM* OOCYSTS

Table A-1 Oocysts dose-response data for oocysts batch no. 1

Trial No.	Age of oocysts (days)	Oocyst dose	No. of wells inoculated	No. of wells infected
2	7	200	4	4
4	7	150	4	4
8	7	100	4	3
11	7	50	4	3
16	7	10	4	1
3	14	200	4	4
5	14	150	4	3
9	14	100	4	3
12	14	50	4	3
17	14	10	4	1
19	14	1	4	0
7	67	125	8	6
10	67	75	8	5
13	67	50	4	0
15	67	25	8	1
18	67	10	8	1
14	68	50	3	2
6	92	150	8	6
1	112	200	8	8

Table A-2 Oocysts dose-response data for oocysts batch no. 2

Trial No.	Age of oocysts (days)	Oocyst dose	No. of wells inoculated	No. of wells infected
1	56	200	8	8
3	56	150	8	7
6	56	100	8	6
16	56	25	8	3
18	56	10	8	0
12	64	50	8	4
4	74	150	8	8
7	74	100	8	6
10	74	50	8	3
2	78	200	8	8
8	78	100	8	5
14	78	25	8	4
5	85	150	8	6
11	85	50	8	5
15	85	25	8	3
17	85	10	8	1
9	111	100	8	7
13	111	50	8	5

APPENDIX B INFORMATION FROM THE FILTRATION RUNS

Trace test on the filter column

Tracer test on the filter column was conducted in order to identify the best sampling time for optimizing the recovery of C. *parvum* oocysts in filtered water. Before conducting the tracer test, the sand-anthracite media filter column was backwashed and flushed with DI water. Two liters methelyne blue (150 mg/L) solution was filtered through the filter column. Absorbance of the filtrate at 664 nm was measured using a spectrophotometer with flow cell attachment and presented in figure B-1. The initial flow rate of methelyne blue solution through the filter column was 6.5 m/h and flow through the spectrophotometer was 3.3 m/h.

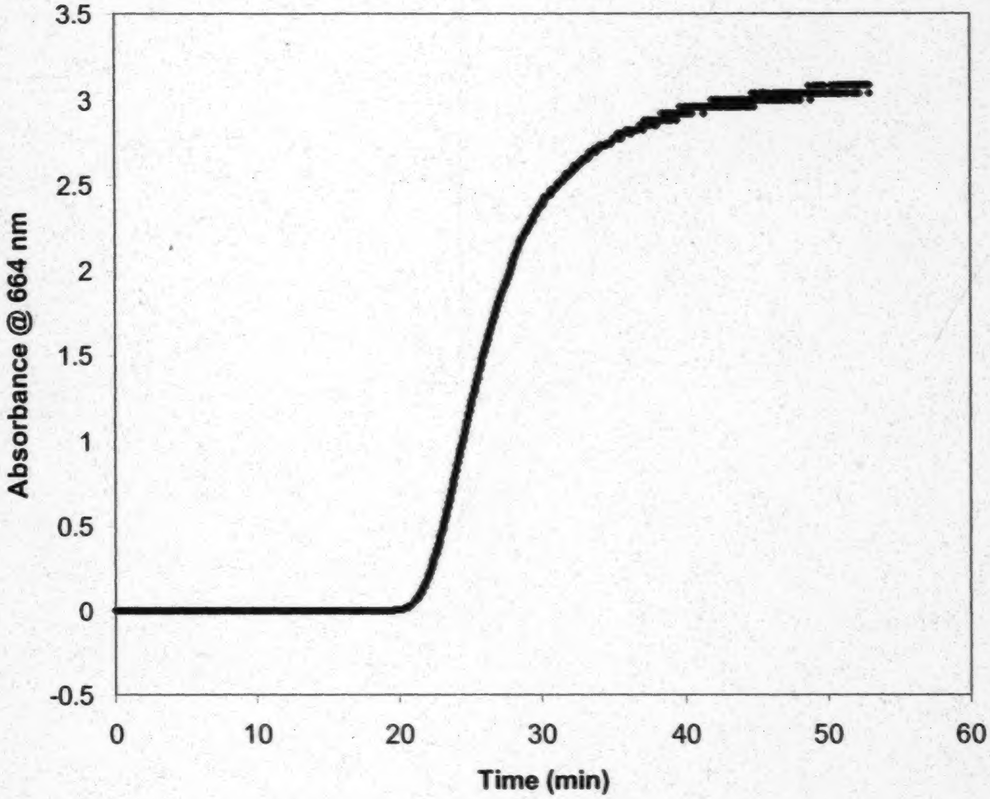


Figure B-1 **Tracer test on filter column using methylene blue**

Data from filtration runs

Table B-1 Turbidity, pH and temperature of water measured at different point during filtration run #1

Point of measurement	Turbidity[1]
Raw water	43.05
Raw water after seeding with oocysts (100 × 10^6)	45.04
Settled water (seeded)	6.89
settled water (unseeded)	7.12
Filtered water (seeded)	1.05

[1] Average of minimum 5 measurements

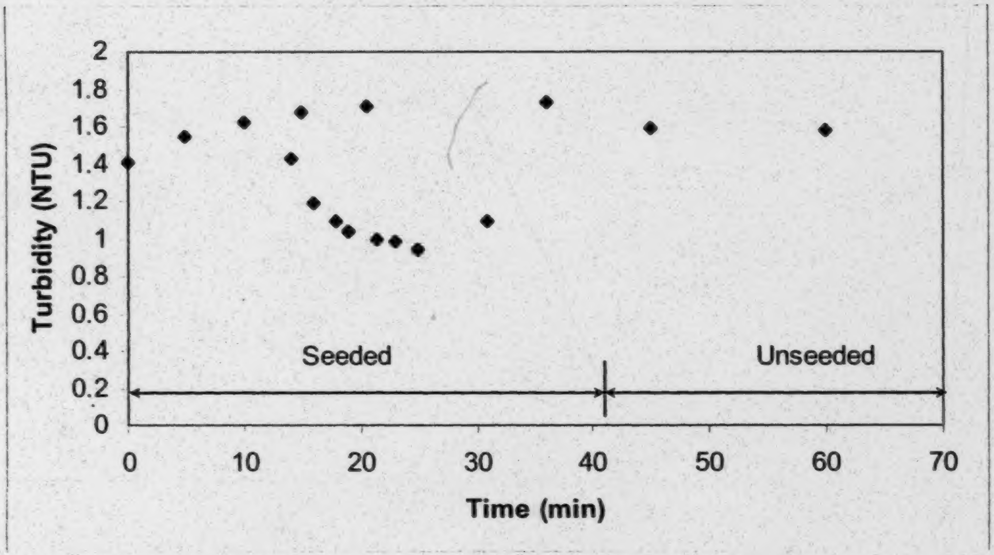


Figure B.2 Turbidity of the filtered water as function of time for filtration run # 1

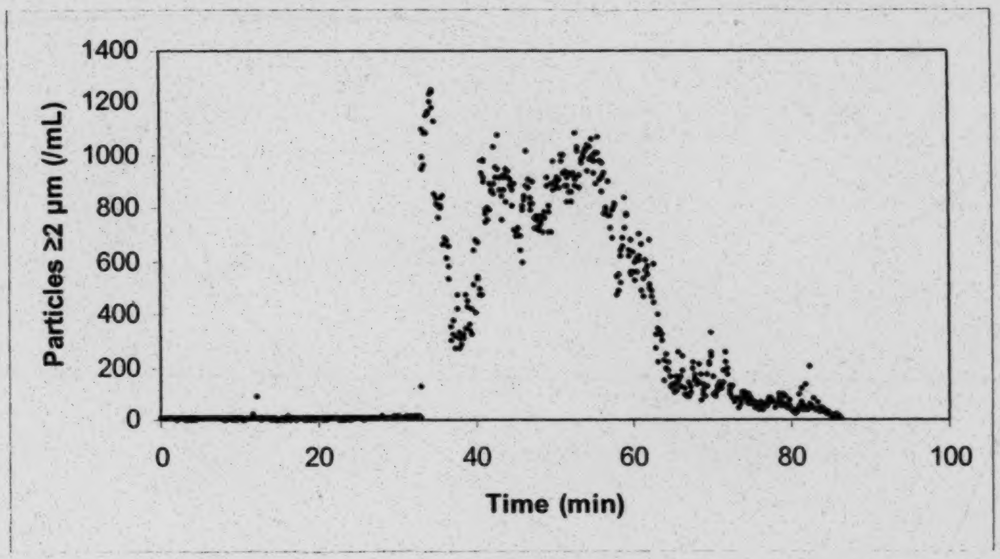


Figure B.3 Particle concentration of the filtered water as function of time for filtration run #1

Table B-2 Turbidity, pH and temperature of water measured at different point during filtration run #2

Point of measurement	Turbidity[1]
Raw water	41.38
Raw water after seeding with oocysts (200 × 10^6)	45.72
Settled water batch 1 (seeded)	7.55
Settled water batch 2 (seeded)	6.9
settled water (unseeded)	9.6
Filtered water (seeded)	1.18
Backwash water	2.17

[1] Average of minimum 5 measurements

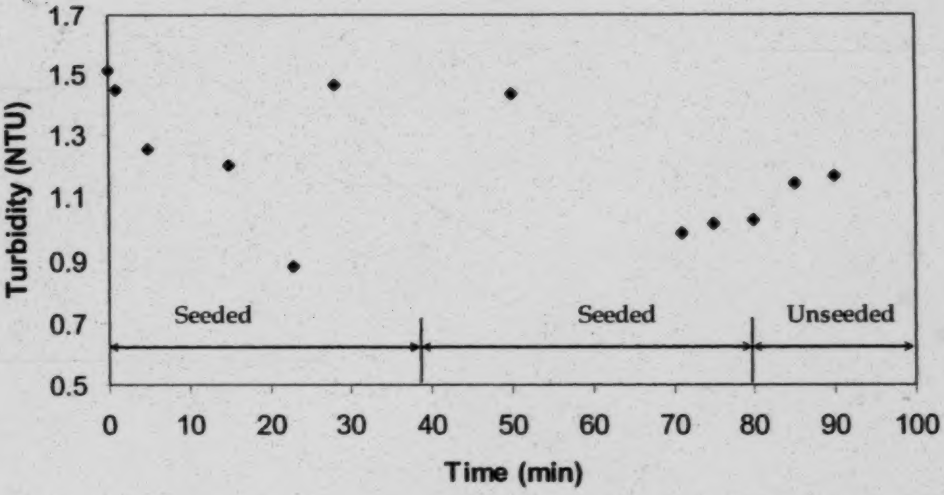


Figure B.4 Turbidity of the filtered water as function of time for filtration run # 2

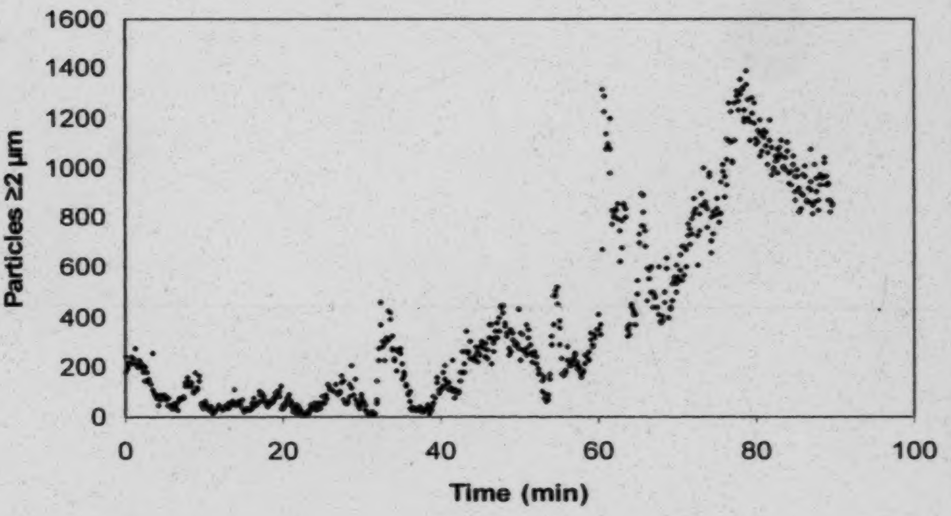


Figure B.5 Particle concentration of the filtered water as function of time for filtration run #2

Table B-3 Turbidity, pH and temperature of water measured at different point during filtration run #3

Point of measurement	Turbidity[1]	pH	Temperature (°C)
Raw water	6.54	7.75	13.4
Raw water after seeding with oocysts (500 × 10^6)	7.93		
Settled water (seeded)	7.60		
settled water (unseeded)	5.95	6.85	15.5
Filtered water (seeded)	1.87		
Filtered water (unseeded)	1.56		

[1] Average of minimum 5 measurements

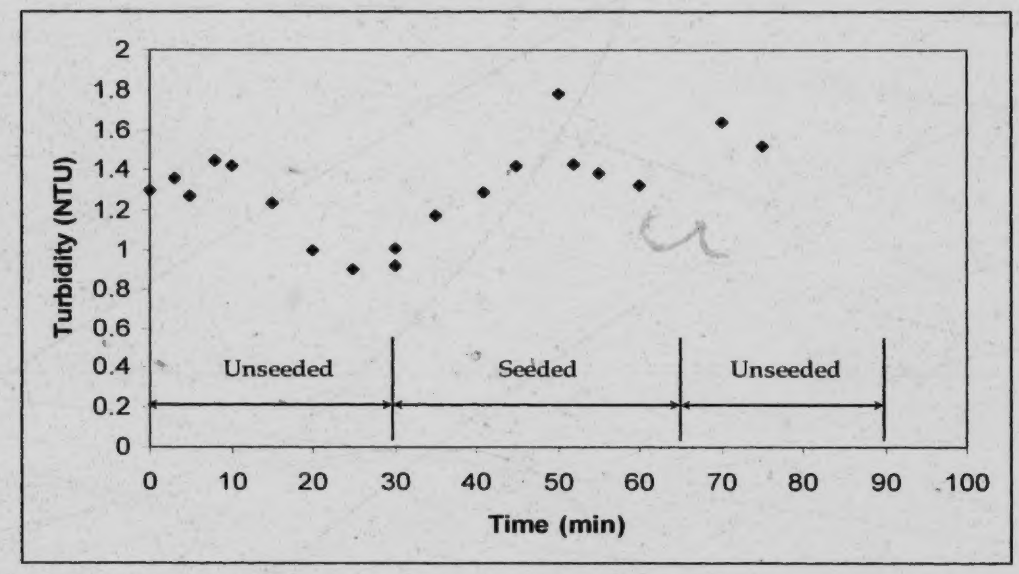


Figure B.6 Turbidity of the filtered water as function of time for filtration run # 3

Table B-4 **Turbidity, pH and temperature of water measured at different point during filtration run #4**

Point of measurement	Turbidity[1]	pH	Temperature (°C)
Raw water	6.21	7.8	9.1
Raw water after seeding with oocysts (500 × 10^6)	8.62		
Settled water (seeded)	8.05		
settled water (unseeded)	5.31	6.5	14.2
Filtered water (seeded)	1.05		
Filtered water (unseeded)	0.96		

[1] Average of minimum 5 measurements

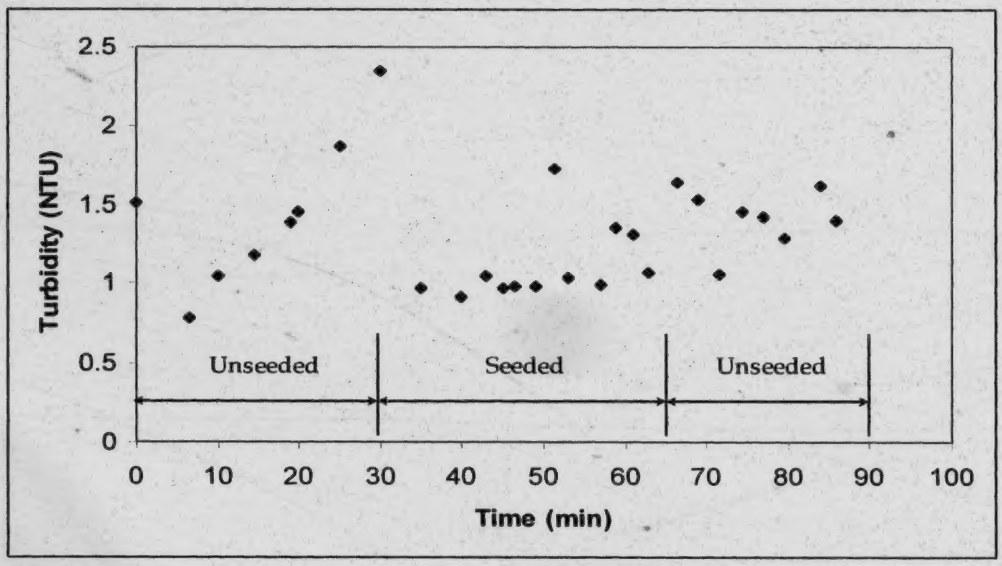


Figure B.7 Turbidity of the filtered water as function of time for filtration run # 4

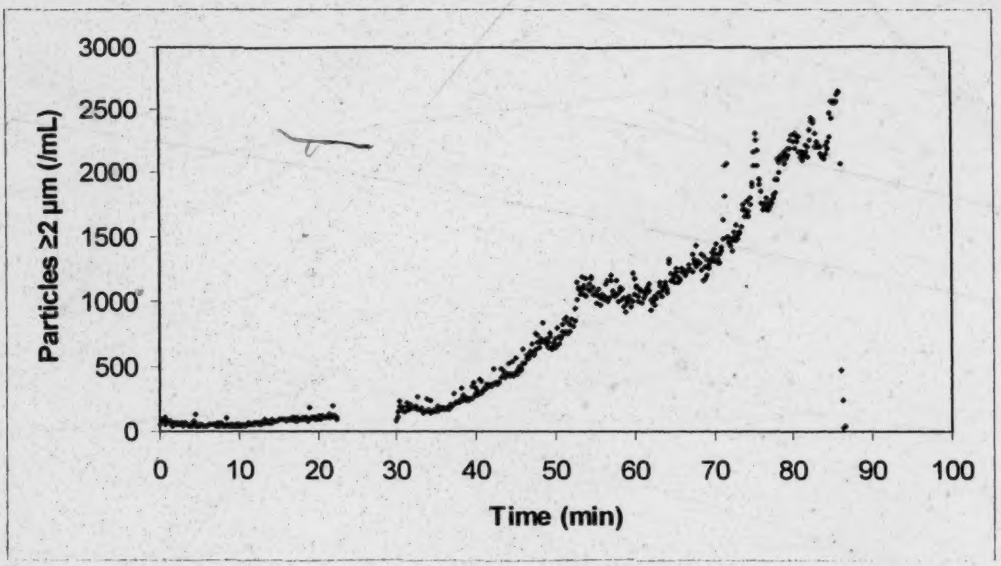


Figure B.8 Particle concentration of the filtered water as function of time for filtration run #4

Table B-5 Turbidity, pH and temperature of water measured at different point during filtration run #5

Point of measurement	Turbidity[1]	pH	Temperature (°C)
Raw water	6.89	7.5	6.4
Raw water after seeding with oocysts (3 × 10^9)	14.37		
Settled water (seeded)	10.6		
settled water (unseeded)	4.67	6.95	13.5
Filtered water (seeded)	3.12		
Filtered water (unseeded)	1.23		

[1] Average of minimum 5 measurements

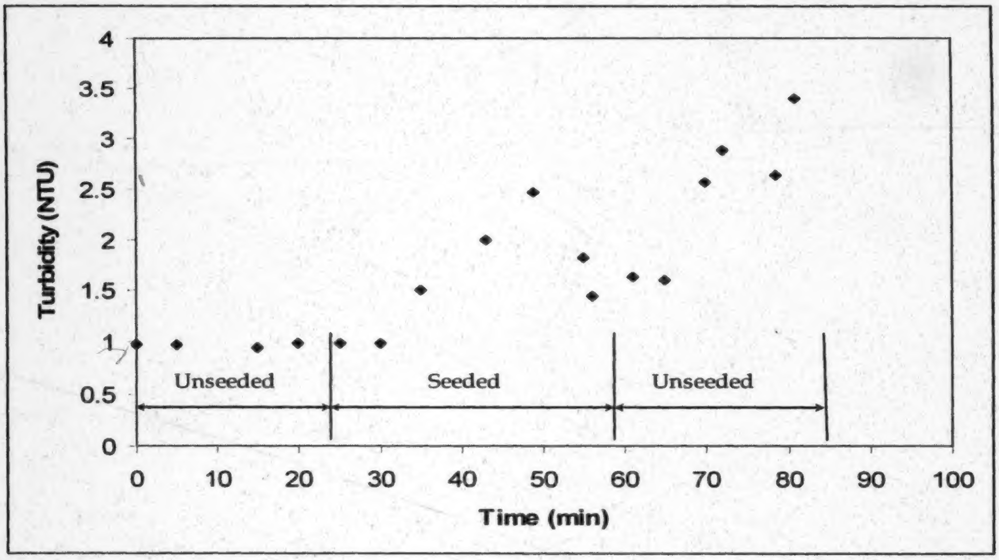


Figure B.9 Turbidity of the filtered water as function of time for filtration run # 5

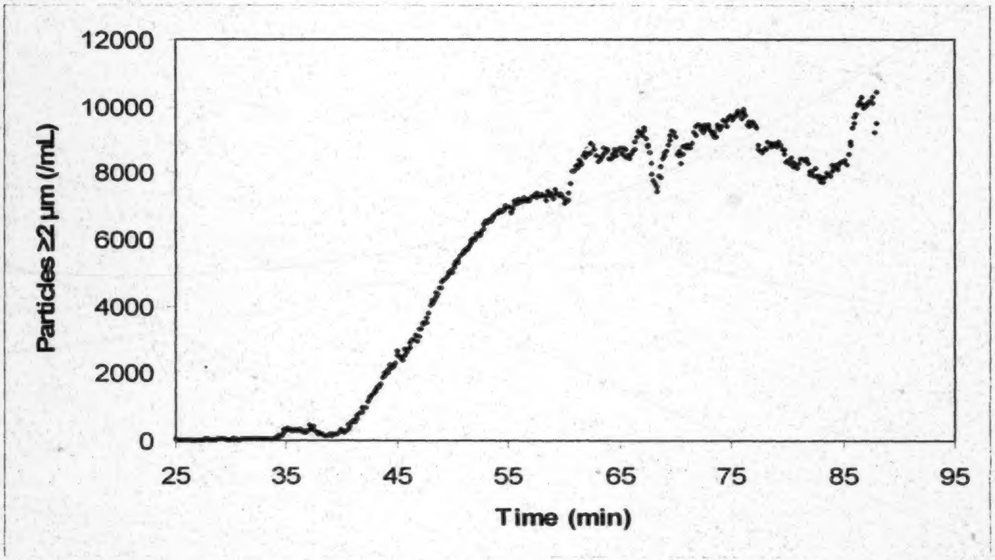


Figure B.10 Particle concentration of the filtered water as function of time for filtration run #5

Table B-6 Turbidity, pH and temperature of water measured at different point during filtration run #6 (November '05 sample)

Point of measurement	Turbidity[1]
Raw water	6.05
Raw water after seeding with oocysts (500 × 10^6)	6.85
Settled water (seeded)	6.22
Filtered water (seeded)	1.36

[1] Average of minimum 5 measurements

Table B-7 Turbidity, pH and temperature of water measured at different point during filtration run #6 (July '05 sample)

Point of measurement	Turbidity[1]
Raw water	44.06
Raw water after seeding with oocysts (500 × 10^6)	44.92
Settled water (seeded)	9.6
Filtered water (seeded)	1.39

[1] Average of minimum 5 measurements

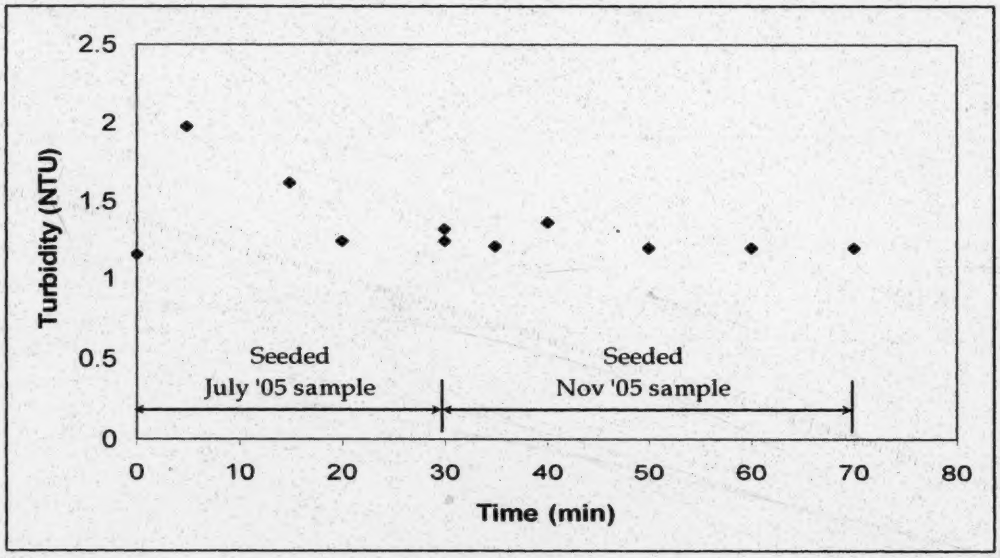


Figure B.11 Turbidity of the filtered water as function of time for filtration run # 6

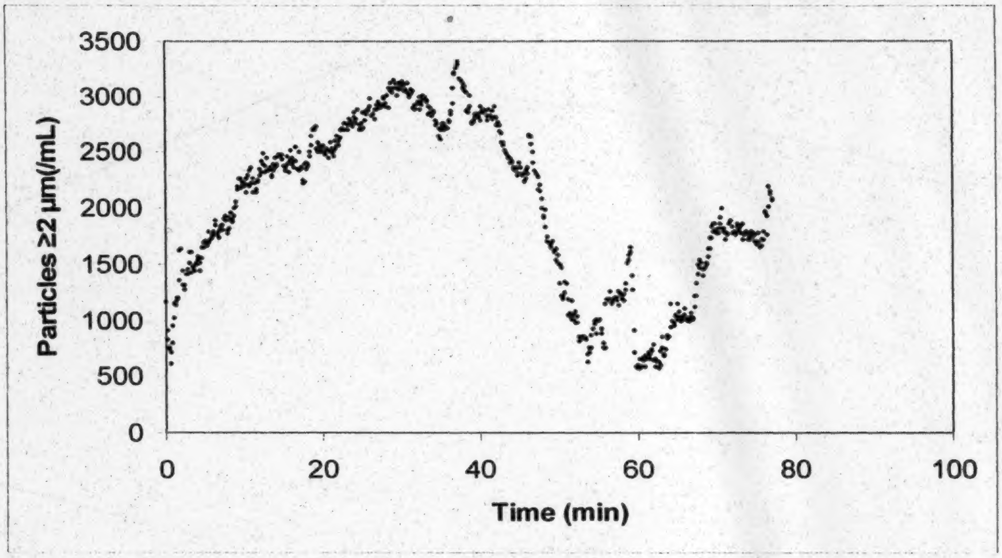


Figure B.12 Particle concentration of the filtered water as function of time for filtration run #6

APPENDIX C INFORMATION FROM THE UV EXPOSURE EXPERIMENTS

Table C-1 **Information from UV exposure trials for oocysts suspended in phosphate buffered water. All the trials were carried out in Petri dishes.**

Trial no	Absorption coefficient[1] (cm^{-1})	Dia of the Petri dish (cm)	Distnce from lamp to the liquid surface (cm)	Radiometer reading at the centre of the Petri dish (mW/cm^2)	*PF*	*RF*	*WF* × *DF*[2]
1	0.047	4.82	41.6	0.083	0.951	0.975	0.922
2	0.041	4.84	41.0	0.083	0.951	0.975	0.929
3	0.0450	4.79	40.8	0.083	0.957	0.975	0.924
4	0.0440	4.79	47.4	0.060	0.982	0.975	0.928
5	0.0560	4.79	48.3	0.063	0.977	0.975	0.916
6	0.0520	4.83	51.4	0.043	0.962	0.975	0.921
7	0.0600	4.84	51.0	0.043	0.962	0.975	0.913
8	0.0570	4.82	50.7	0.044	0.944	0.975	0.916
9	0.0520	4.82	50.9	0.044	0.952	0.975	0.921
10	0.0550	4.82	50.7	0.044	0.944	0.975	0.918

[1] Measured at 254 nm with a regular transmittance spectrophotometer

[2] Calculated together using numerical integration

Table C-2 **Conditions of the UV exposure trials for oocysts collected in filtered water from filtration run no. 3. All the trials were carried out in Petri dishes.**

Trial no	Sample type	Absorption coefficient[3] (cm^{-1})	Dia of the Petri dish (cm)	Distance from lamp to the liquid surface (cm)	Radiometer reading at the centre of the Petri dish (mW/cm^2)	*PF*	*RF*	*WF* × *DF*[4]
1	UV+H[1]	0.0690	4.88	46.2	0.053	0.898	0.975	0.901
2	UV+H[1]	0.0760	4.75	46.2	0.053	0.898	0.975	0.894
3	UV+H[1]	0.0695	4.79	46.2	0.053	0.898	0.975	0.900
4	H+UV[2]	0.0665	4.76	46.2	0.053	0.898	0.975	0.904
5	H+UV[2]	0.1060	4.85	46	0.053	0.898	0.975	0.863
6	H+UV[2]	0.0700	4.75	45.8	0.053	0.898	0.975	0.899

[1] Filtered water homogenized after UV exposure

[2] Filtered water homogenized after UV exposure

[3] Measured at 254 nm by a spectrophotometer with integrating sphere attachment

[4] Calculated together using numerical integration

Table C-3 **Conditions of the UV exposure trials for oocysts collected in filtered water from filtration run no. 4. All the trials were carried out in Petri dishes.**

Trial no	Sample type	Absorption coefficient[3] (cm^{-1})	Dia of the Petri dish (cm)	Distance from lamp to the liquid surface (cm)	Radiometer reading at the centre of the Petri dish (mW/cm^2)	*PF*	*RF*	*WF* × *DF*[4]
1	UV+H[1]	0.0870	4.8	45.5	0.054	0.880	0.975	0.882
2	UV+H[1]	0.0840	4.85	45.5	0.053	0.870	0.975	0.885
3	UV+H[1]	0.0805	4.75	45.5	0.054	0.880	0.975	0.889
4	H+UV[2]	0.0895	4.75	46.0	0.053	0.870	0.975	0.880
5	H+UV[2]	0.0945	4.80	45.5	0.053	0.870	0.975	0.874
6	H+UV[2]	0.0960	4.79	46.0	0.054	0.880	0.975	0.873

[1] Filtered water homogenized after UV exposure

[2] Filtered water homogenized after UV exposure

[3] Measured at 254 nm by a spectrophotometer with integrating sphere attachment

[4] Calculated together using numerical integration

Table C-4 Information from UV exposure trials for oocysts collected in filtered water from filtration run no. 5. All the trials were carried out in 150 mL beakers.

Trial no	Sample type	Absorption coefficient[2] (cm^{-1})	Dia of the beaker (cm)	Distance from lamp to the liquid surface (cm)	Radiometer reading at the centre of the beaker (mW/cm^{2})	*PF*	*RF*	*WF* × *DF*[4]
1	UV+H[1]	0.159	5.43	44	0.058	0.841	0.975	0.328
2	UV+H[1]	0.168	5.43	44.5	0.057	0.849	0.975	0.336
3	UV+H[1]	0.163	5.43	44	0.058	0.841	0.975	0.323
4	H+UV[2]	0.165	5.43	44	0.058	0.841	0.975	0.320
5	H+UV[2]	0.167	5.43	44	0.058	0.841	0.975	0.317
6	H+UV[2]	0.171	5.43	44	0.059	0.830	0.975	0.311

[1] Filtered water homogenized after UV exposure

[2] Filtered water homogenized after UV exposure

[3] Measured at 254 nm by a spectrophotometer with integrating sphere attachment

[4] Calculated together using numerical integration

APPENDIX D STATISTICAL ANALYSIS

Table D-1 **Summary output of ANOVA for filtered water samples exposed at 5 mJ/cm²**

Anova: Two-Factor With Replication

SUMMARY	**Filtration run #3**	**Filtration run #4**	**Total**
UV+H			
Count	3	3	6
Sum	5.220	4.648	9.867
Average	1.740	1.549	1.645
Variance	0.480	0.099	0.243
H+UV			
Count	3	3	6
Sum	7.598	6.887	14.485
Average	2.533	2.296	2.414
Variance	0.090	0.218	0.140
Total			
Count	6	6	
Sum	12.817	11.535	
Average	2.136	1.922	
Variance	0.417	0.294	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	1.777	1	1.777	8.010	0.022	5.318
Columns	0.137	1	0.137	0.618	0.454	5.318
Interaction	0.002	1	0.002	0.007	0.934	5.318
Within	1.774	8	0.222			
Total	3.690	11				

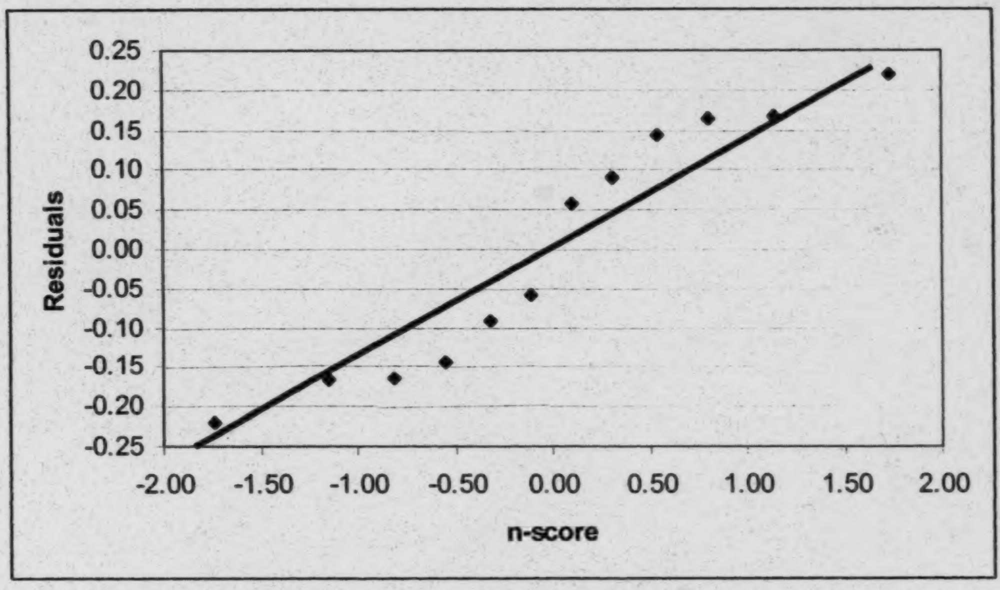


Figure D.1 Normal probability plot of residuals for ANOVA at 5 mJ/cm²

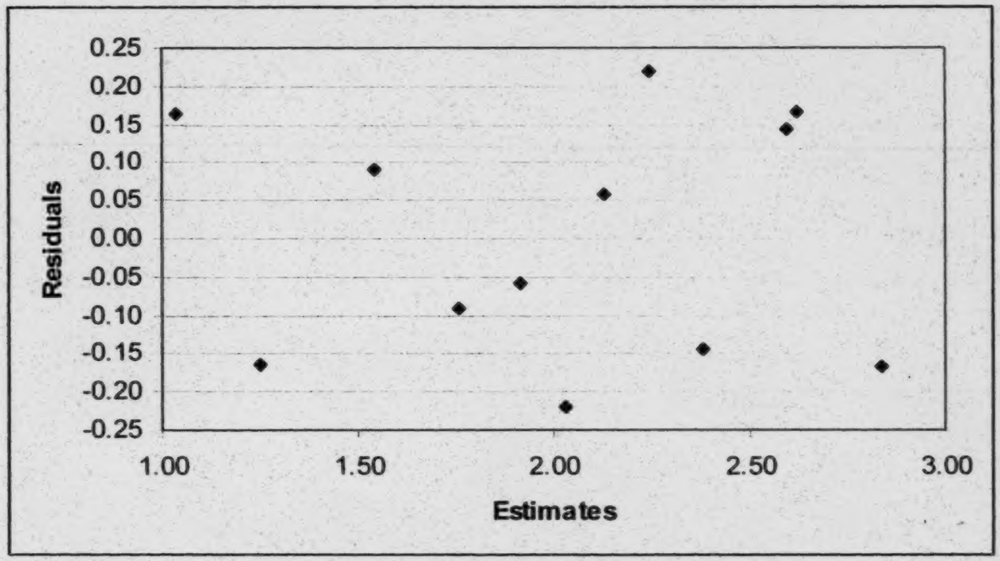


Figure D.2 Plot of residuals vs estimates for ANOVA at 5 mJ/cm²

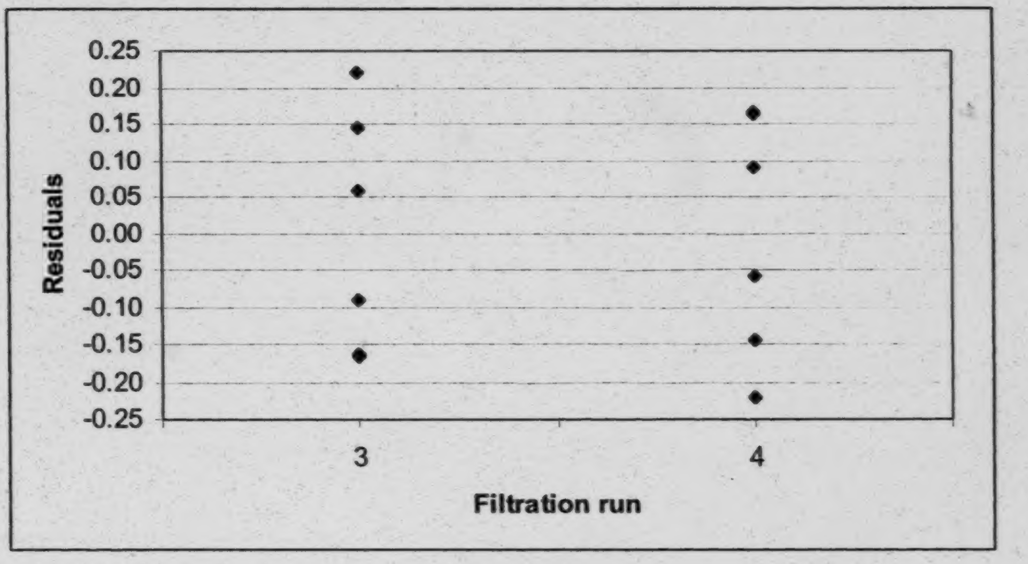


Figure D.3 Plot of residuals vs filtration run for ANOVA at 5 mJ/cm²

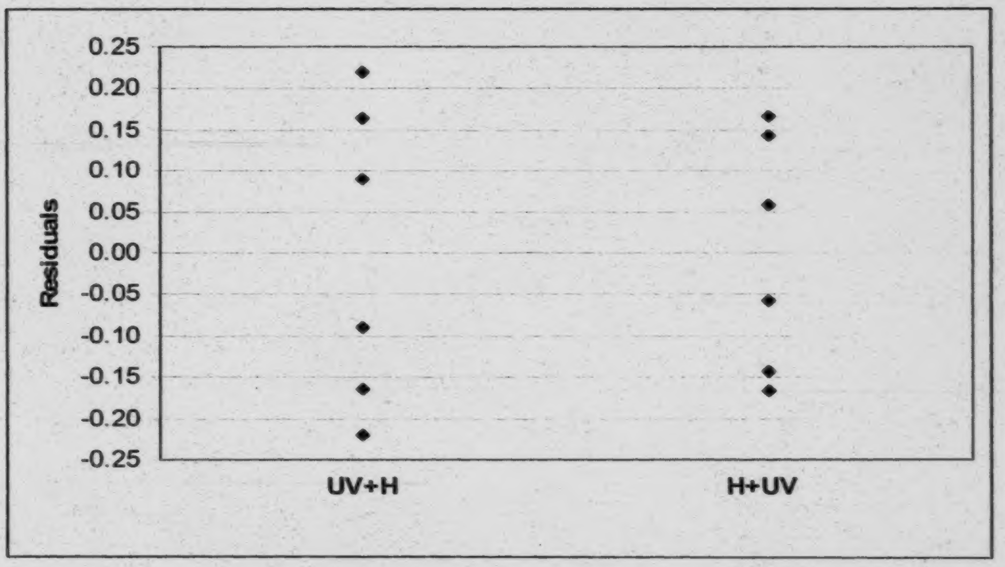


Figure D.4 Plot of residuals vs sample type for ANOVA at 5 mJ/cm²

Table D-2 Summary output of ANOVA for filtered water exposed at 40 mJ/cm²

Anova: Single Factor (alpha = .05)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
UV+H	3	9.049	3.016	0.148
H+UV	3	12.248	4.083	0.408

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.705	1	1.705	6.125	0.069	7.709
Within Groups	1.114	4	0.278			
Total	2.819	5				

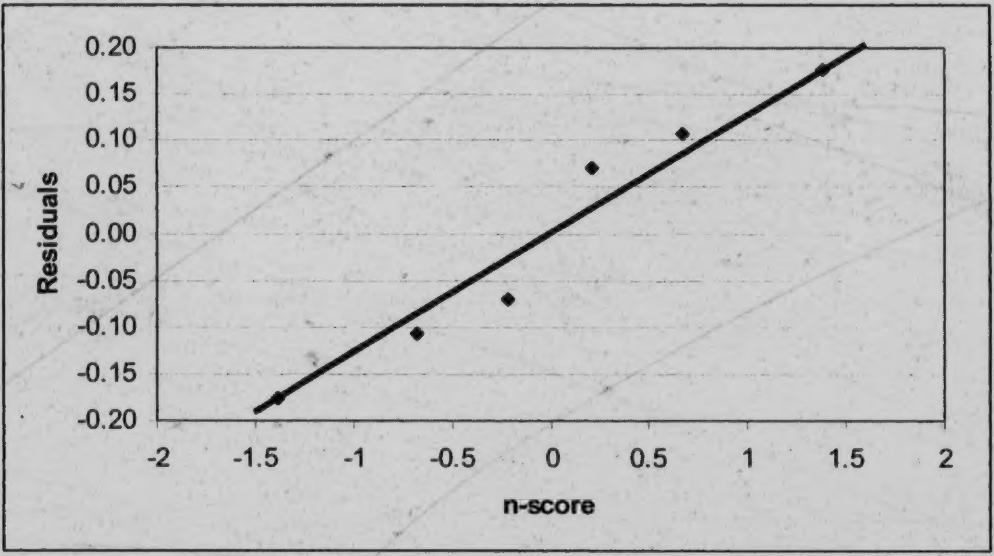


Figure D.5 Normal probability plot of residuals for ANOVA at 40 mJ/cm²

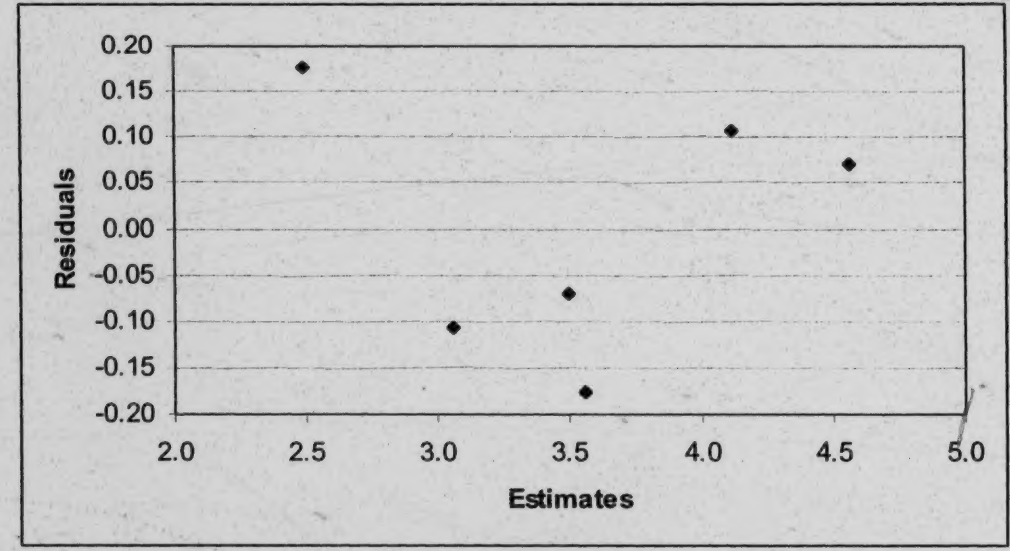


Figure D.6 plot of residuals vs estimates for ANOVA at 40 mJ/cm²

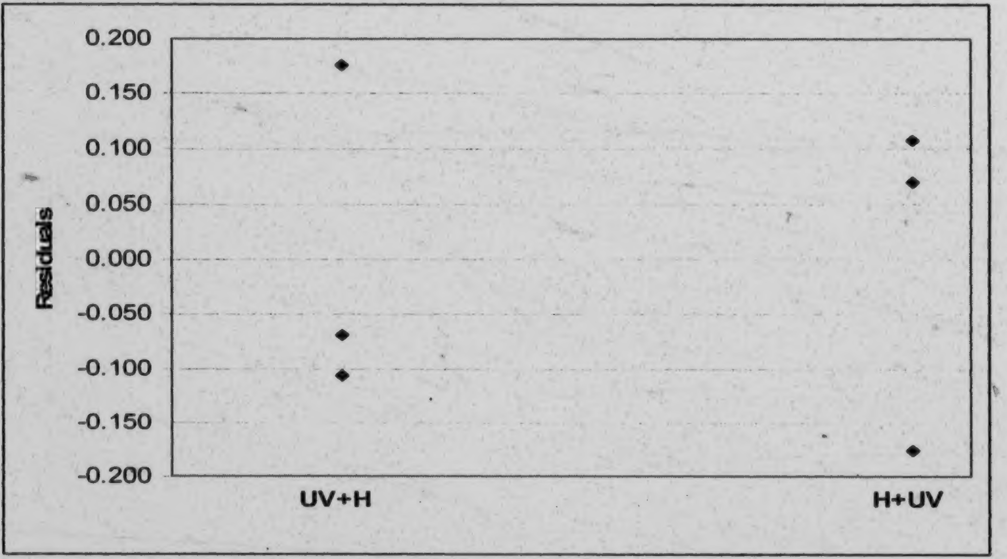


Figure D.7 Plot of residuals vs sample type for ANOVA at 40 mJ/cm²

APPENDIX E DATA FROM CUT-OFF FILTRATION EXPERIMENTS AND IMAGES FROM SCANNING ELECTRON AND CONFOCAL MICROSCOPY

Table E-1 Data from cut-off filtration experiment

Filter size (µm)	Size fraction (µm)	Sample type	Average oocyst Count ($\times 10^4$)	% of total oocysts retained on filter	% of total recovered oocysts retained on filter
10	≥10	Filtered water - 1	0.0875	0.11	0.20
		Filtered water - 2	3.54	5.24	22.43
		Backwash water-2	4.1	4.44	74.74
		Filtered water - 6	39.31	3.70	13.02
		Clean suspension	1.425	0.97	1.90
5	≥ 5 ≤ 10	Filtered water - 1	36.31	47.06	84.09
		Filtered water - 2	8.5	12.59	53.85
		Backwash water-2	1.15	1.24	20.96
		Filtered water - 6	185.72	17.47	61.50
		Clean suspension	12.01	8.21	16.05
2	≥ 2 ≤ 5	Filtered water - 1	4.52	5.86	10.47
		Filtered water - 2	3.62	5.36	22.93
		Backwash water-2	0.2035	0.22	3.71
		Filtered water - 6	55.5	5.22	18.38
		Clean suspension	60.29	41.22	80.55
0.8	≥ 0.8 ≤ 2	Filtered water - 1	2.26	2.93	5.23
		Filtered water - 2	0.125	0.19	0.79
		Backwash water-2	0.032	0.03	0.58
		Filtered water - 6	21.44	2.02	7.10
		Clean suspension	1.121	0.77	1.50

Table E-2 Oocysts recovery from each sample

Sample type	Total number of oocyst (×10^4)	Oocysts recovered	Percent recovery
Filtered water - 1	77.15	43.18	55.97
Filtered water - 2	67.5	15.79	23.39
Backwash water-2	92.37	5.49	5.94
Filtered water - 6	1063	301.97	28.41
Clean suspension	146.25	74.85	51.18

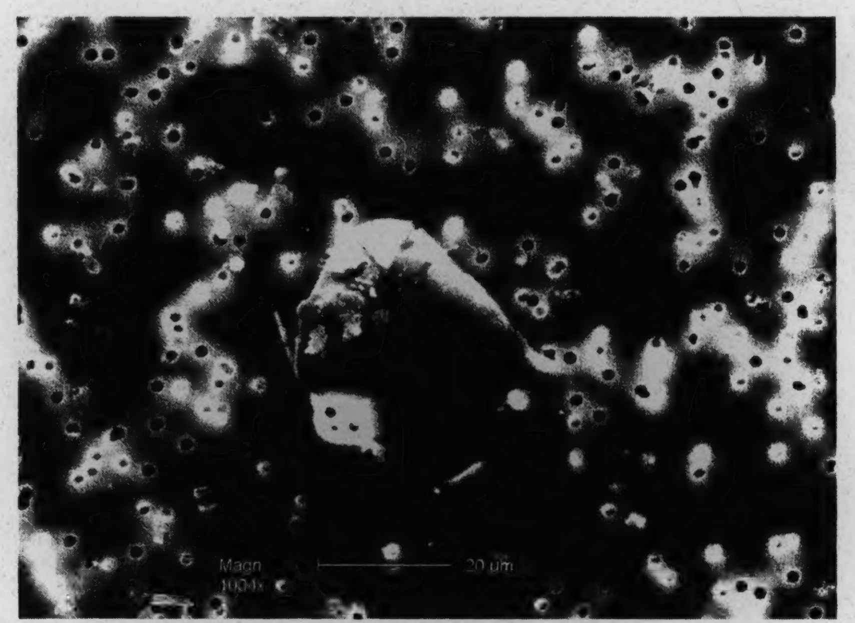


Figure E.1 SEM micrograph showing a large particle captured on a 5 µm pore size PCTE filter from backwash water

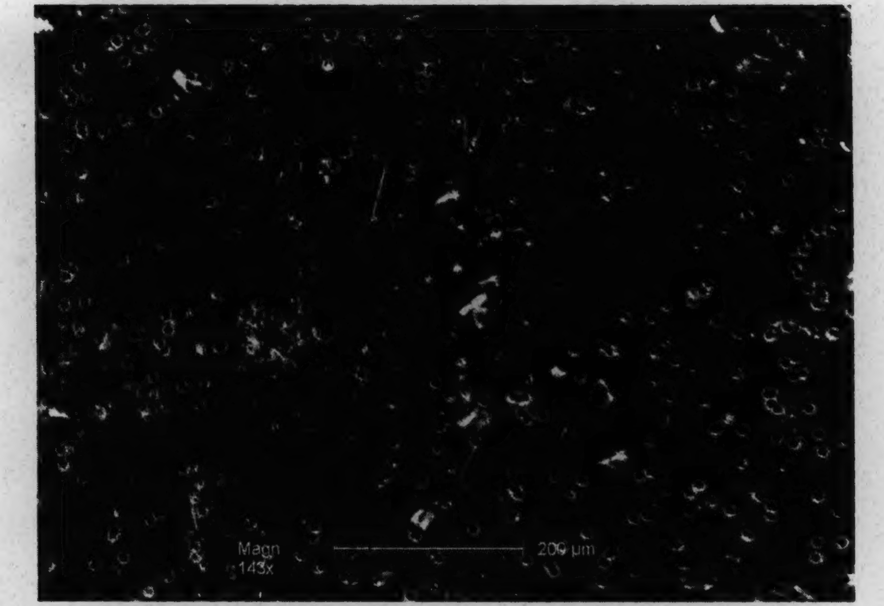

Magn
143x
200 µm

Figure E.2 SEM micrograph showing particles captured on a 10 µm pore size PCTE filter from backwash water

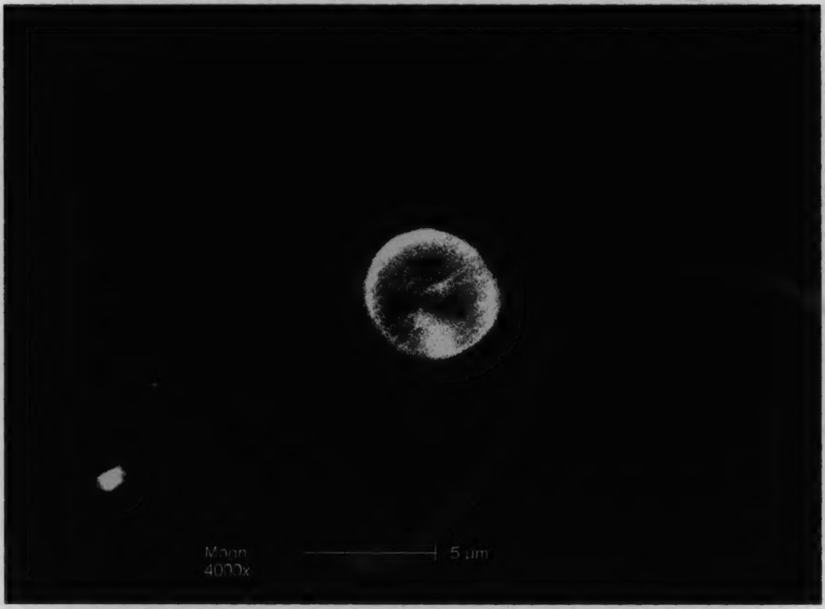

Magn
4000x
5 µm

Figure E.3 SEM micrograph of an isolated *C. parvum* oocyst captured on a 0.8 µm pore size PCTE filter

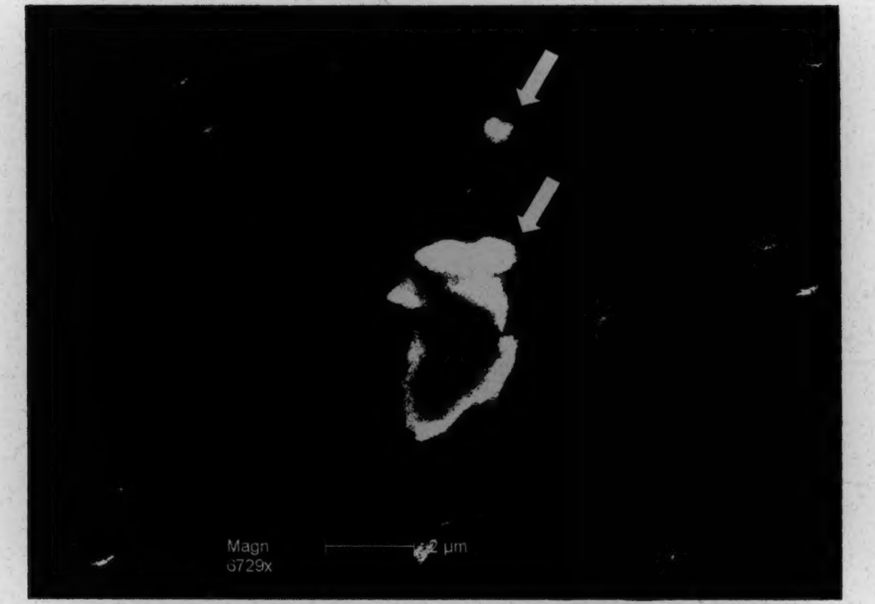


Figure E.4 SEM micrograph of a floc particle with *C. parvum* oocysts-like particle attached to the surface of the floc particle. The pore size of the PCTE filter was 5 µm.

Figure E.5 DIC-IFA image of aggregated *C. parvum* oocysts captured from filtered water. Oocysts appear to be associated with large floc particles

Figure E.6 DIC-IFA image of a floc particle captured form filtered water. *C. parvum* oocysts appear to be attached to the surface of the floc particle

Figure E.7 DIC-IFA image of a particle captured form filtered water. *C. parvum* oocysts appear to be attached to the surface of the particle